Fatty Acid Oxidation and Chlorophyll Bleaching

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Lipoxygenase (linoleate : oxygen oxidoreductase ; E.C. 1.13.1.13 : also called lipoxidase) catalyzes the oxidation of certain polyunsaturated fatty acids with *cis, cis*-1,4-pentadiene unit such as linoleic and linolenic acids. Fatty acids are oxidized to their respective conjugated diene hydroperoxides in the presence of molecular oxygen. Lipoxygenase activity has been reported in many higher plants, mainly leguminous seed, cereal grains, and oil seeds. Potato tubers[1] and leaves of some plants[2,3] were also found to be active. Recently, Zimmerman and Vick[4] observed the activity of lipoxygenase in *Chlorella pyrenoidosa* when cells were homogenized under anaerobic conditions.

The bleaching of carotene in the presence of polyunsaturated fatty acids *in vitro* has also been known for many years. While Sumner and Sumner[5] reported that lipoxygenase and "carotene oxidase" were identical, Kies *et al.*[6] observed that homogeneous crystalline lipoxygenase of soybean was essentially ineffective in bleaching carotene. Presence of a heat-sensitive entity which was distinct from Theorell enzyme[7] was required for bleaching carotene under their experimental conditions.

In 1965, Holden[8] reported the enzymatic bleaching of Chl in the presence of long chain fatty acids with leguminous seed extracts. She confirmed that Chl bleaching was coupled with a chain reaction involving peroxidation of fatty acid by lipoxygenase and subsequent breakdown of hydroperoxide by a heat-labile factor. Later on, a new enzyme which catalyzes the isomerization of hydroperoxides formed by lipoxygenase was isolated from flaxseed by Zimmerman and Vick[9,10], and named as hydroperoxide isomerase. Since Chl was not bleached when this enzyme was inactivated by heating, they proposed that the heat-labile factor described by Holden corresponded to hydroperoxide isomerase.

Little is known about the mechanism of Chl degradation *in vivo* during senescence. The present study was initiated regarding the

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Abbreviations: EDTA, disodium ethylenediaminetetraacetic acid; Chl, chlorophyll; DIECA, sodium diethyldithiocarbamate; NDGA, nordihyroguaiaretic acid.

lipoxygenase-catalyzed bleaching of Chl as one of the model system of Chl degradation. A possible involvement of lipoxygenase-linoleate system in the Chl degradation *in vivo* is presumable from the following findings: (i) During senescence, a rapid decline in some complex lipid levels, particularly those characteristic of chloroplasts, is observed[11]. (ii) Chloroplasts are rich source of unsaturated fatty acids, especially linolenic acid[12]. (iii) The 1st step of disappearance of cellular lipid during senescence might involve the degradation of endogenous lipid by lipase to yield free fatty acids and water soluble components, and the former are broken down catabolically to CO_2. Possible involvement of lipoxygenase in this catabolism is supposed by the observation that linolenic acid levels fall much faster than those of other acids during senescence[13]. Preliminary experiments were attempted to determine the involvement of any factors such as hydroperoxide isomerase besides lipoxygenase in the bleaching of Chl and some attempts were made to cause senescence in wheat and barley leaves and the changes in their lipoxygenase activity were measured.

Materials and Methods

Plant materials

Seeds of wheat (*Triticum aestivum* cv. Norin No. 26), barley (*Hordeum vulgare* cv. Kikaihadaka), oat (*Avena sativa* cv. Victory), rice (*Oryza sativa*), azuki bean (*Vigna angularis*), black gram (*Phaseolus mungo*), and pea (*Pisum sativum*) were grown on a moistened filter paper in the light at room temperature. Potato (*Solanum tuberosum*) was grown in the field.

Preparation of substrates

(Fatty acid solutions) Aqueous solution of linoleic and linolenic acids was prepared essentially by the method of Grossman *et al.*[3] Linoleate hydroperoxides were prepared as follows; incubation mixture contained 2 ml of linoleic acid solution and lipoxygenase in 120 ml of buffer (50 mM sodium borate, pH 9.0, for soybean enzyme or 50 mM sodium phosphate, pH 6.8, for potato), and was incubated for 20 min at 25 C with constant shaking. After incubation the mixture was acidified to pH 3.0 and extracted with diethyl ether. The ether extract was dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was chromatographed on thin layer of Silica Gel G (Merck) with the solvent system of isooctane-ether-acetic acid (50 : 50 : 1, v/v). The hydroperoxide were detected with 0.1% N, N-dimethyl-*p*-phenylene diamine in chloroform-acetic acid-water (5 : 5 : 1, v/v), scraped off, eluted with 50% ethanol, and used as substrate for hydroperoxide isomerase. This substrate was freshly prepared for each experiment.

(Chlorophylls and their derivatives) Chls *a* and *b* prepared from the acetone extract of spinach leaves were dissolved in 50 mM phosphate buffer, pH 6.8, containing 0.2% Triton X-100, respectively. Pheophytin *a* was prepared from Chl *a* by the procedure of McFeeters *et al.*[14]. Ethyl chlorophyllide *a* was prepared from fresh *Ailanthus altissima* leaves according to the method of Holt and Jacobs[15]. Ethyl pheophorbide *a* was prepared by adding 1 N HCl to ethyl chlorophyllide *a*. After conversion was completed, petroleum ether was added to give a final ether concentration of 10%. The solution was washed with water to remove HCl and acetone. Zn-pheophytin *a* was prepared from pheophytin *a* by the method of Sherman *et al.*[16]. Those derivatives were dissolved in 50 mM phosphate buffer, pH 6.8, containing 0.2% Triton X-100 and used as substrates.

Preparation of enzyme

(Extraction) Leaves of cereal grains were snipped at the base and cut into fine pieces. These pieces were mixed with 50 mM Tris-HCl buffer, pH 7.2, and homogenized in a blendor at maximum speed in two 30 sec bursts with 30 sec interval. The homogenate was squeezed through two layers of cotton gauze and centrifuged at 20,000×g for 20 min at 0°C. The resulting supernatant was used as a crude extract. Potato tubers were surface sterilized with 0.1% sodium hypochlorite and rinsed throughly with running tap water. Leaves and sterilized tubers of potato were minced, and homogenized with the same buffer containing 2 mM $Na_2S_2O_5$ to prevent the oxidation of phenolic substances. Ungerminated soybean was crushed finely into flake and defatted successively with acetone, acetone-petroleum ether (1 : 1, v/v), and petroleum ether. After drying, the defatted flake was homogenized with 0.1 M acetate buffer, pH 4.5.

(Purification) The crude extract was saturated to 50% with solid $(NH_4)_2SO_4$, and the preciptate thus formed was taken up with 10 mM phosphate buffer, pH 6.8 and dialyzed against the same buffer. After removal of insoluble material by centrifugation, the dialyzate was applied on a column of DEAE-Sephadex (2.5×2.0 cm), equilibrated with the dialysis buffer. The active fraction was eluted with the same buffer containing 0.17 M NaCl, concentrated by ultra-filtration (Diaflo ; M.W. 10,000 cut off), and dialyzed against 10 mM Tris-HCl, pH 7.2. The dialyzed solution was charged to a column of Sephadex G-200 (2.5×95 cm), which was equilibrated and eluted with the dialysis buffer. The fractions that contained the lipoxygenase activity were pooled and used for further study. In the case of the purification of soybean enzyme, the fraction obtained at 35-56% saturation with $(NH_4)_2SO_4$ was subjected to gel filtration on Sephadex G-150 column (2.5×95 cm) using 10 mM Tris-HCl, pH 7.2. Active fractions were further applied on DEAE-Sephadex column (1.5×25 cm) previously equilibrated with

the above buffer. Elution was continued successively with the same buffer containing 0.1 M and 0.2 M NaCl. Most of lipoxygenase activity was eluted with the buffer containing 0.2 M NaCl.

Assays

Activities of linoleate oxidation by lipoxygenase and hydroperoxide decomposition by hydroperoxide isomerase were determined by measuring the absorption at 234 nm due to conjugated diene hydroperoxides[17], and the activity of Chl bleaching at 668 nm.

The reaction cuvette contained, unless otherwise noted, 3 ml of 50 mM phosphate buffer (pH 6.8), 0.05 ml of linoleic acid solution (2.7×10^{-2} M), 0.05 ml of Chl *a* solution (3.0×10^{-4} M), and 0.05 ml of enzyme. In the assay for hydroperoxide isomerase, linoleic acid solution was replaced with the same volume of linoleate hydroperoxide solution (3.2×10^{-3} M). Peroxidase was determined by measuring the increase at 470 nm, with guiacol as hydrogen donor[18]. Catalase was assayed by the decrease of absorbance due to H_2O_2 at 240 nm[19]. The measurement was carried out at room temperature and the rate of reaction was calculated from the linear portion of the curve. One enzyme unit is defined as the amount of enzyme which produces a change in absorption of 1.0 per min. Pretein was measured by the method of Lowry *et al.*[20] and Chl was determined spectrophotometrically[21].

Product analysis

The analysis of the reaction products of wheat lipoxygenase was done as follows; Three hundred ml of 50 mM phosphate buffer (pH 6.5), 5 ml of linoleic acid solution, and 5 ml of partially purified wheat lipoxygenase were mixed and incubated for 30 min at 25°C with constant shaking. The reaction mixture was adjusted to pH 9.0 and 50 mg of $NaBH_4$ was added in order to convert the hydroperoxides to corresponding hydroxy compounds. After stirring for 1 hr at 25°C, the solution was acidified to pH 3.0 and extracted with diethyl ether. The hydroxy fatty acids were then converted to their methyl esters with diazomethane. The resulted methyl esters were chromatographed on Silica Gel F_{254} (Merck) plates using petroleum ether (b.p. 60–80°C)-diethyl ether-acetic acid (70 : 30 : 1, v/v). The developing solvent was renewed each time and the separation of 9- and 13-hydroxy esters was improved by double development. Reaction products of lipoxygenase from soybean and potato tuber were assayed in the same way.

Electrophoresis

Disc gel electrophoresis of protein was performed on 7.5% acrylamide gel using the method of Davis[22]. Electrophoresis was conducted at 1 mA per tube and terminated when the tracking dye had moved 6 cm from the gel top. The gels were stained with Coomassie briliant blue according to Weber and Osborn[23]. Specific staining for lipo-

xygenase was achieved essentially by the method of Verhue and Francke[24].

Results

Chlorophyll bleaching by lipoxygenase

It was observed that extracts of various plant materials actively bleached Chl in the presence of linoleic acid. The bleaching was

Table 1. Substrate specificity of wheat lipoxygenase

Substrate	Specific activity* (ΔA/min mg)	
	Fatty acid oxidation	Chlorophyll bleaching
Linoleic acid ($C_{18:2}$)	11.7	0.20
Linolenic acid ($C_{18:3}$)	16.2	0.28
Oleic acid ($C_{18:1}$)	0	0

* Specific activity: changes in absorption per min per mg protein.

Table 2. Activities of linoleate oxidation, chlorophyll bleaching, and hydroperoxide isomerase of various plants.

Plant source	Specific activity		
	Linoleate oxidation	Chlorophyll bleaching	Hydroperoxide isomerase
Wheat (*Triticum aestivum*)			
Leaves (10-day-old)	1.8	0.05	0.38
Roots (10-day-old)	1.4	0.04	0.45
Barley (*Hordeum vulgare*)			
Leaves (19-day-old)	0.79	0.02	0.25
Oat (*Avena sativa*)			
Leaves (10-day-old)	0.62	0.05	trace
Rice (*Oryza sativa*)			
Leaves (13-day-old)	0.80	trace	0
Corn (*Zea mays*)			
Leaves (10-day-old)	0.3	0.07	5.60
Potato (*Solanum tuberosum*)			
Leaves (young)	3.7	trace	ND*
Tubers (mature)	10.0	0.74	0.02
Soybean (*Glycine max*)	6.5	0.31	0.02
Broad bean (*Vicia faba*)	1.0	0.09	ND
Azuki bean (*Vigna angularis*)	10.3	0.97	ND
Black gram (*Phaseolus mungo*)			
(light-grow)	9.0	0.51	trace
(dark-grown)	5.8	0.48	trace
Pea (*Pisum sativum*)	4.2	0.24	ND

Activities of crude extracts were measured. For details, see text.
* ND, not determined.

proportional to the amount of extract added and was completely suppressed by boiling the extract. Chl was also bleached in the presence of linolenic acid instead of linoleic, but not in the presence of oleic acid which cannot be a substrate for lipoxygenase. The enzyme of crude wheat extract exhibited higher specific activities of fatty acid oxidation and Chl bleaching with linolenic acid as substrate than with linoleic (Table 1). The result shown here suggests that a lipoxygenase-unsaturated fatty acid system may be involved in Chl bleaching.

Crude extracts of various plant sources were tested for their activities of linoleate oxidation, Chl bleaching and hydroperoxide isomerase. As is evident from Table 2, there is little correlations between the activities of Chl bleaching and hydroperoxide isomerase; potato tuber, soybean and black gram did not have significant hydroperoxide isomerase activities but showed considerably high activities in Chl bleaching. On the contrary, leaves of wheat and corn which had rather high hydroperoxide isomerase activities showed low activities in Chl bleaching. Table 2 also shows that the activity of linoleate oxidation is, with a few exceptions, roughly correlated with the activity of Chl bleaching; there is usually little bleaching when the activity of linoleate oxidation is low.

When linoleate hydroperoxide solution was used as substrate, wheat extract did not bleach Chl Addition of linoleic acid to the reaction mixture caused a substantial lossof absorption at 668 nm (Fig. 1b). From these facts. it is probable to suppose that Chl is bleached during the formation of hydroperoxides and not by their decomposition process.

The elution pattern of crude wheat extract from Sephadex G-200 are shown in Fig. 2, which indicates the clear separation of hydroperoxide isomerase from the Chl bleaching activity and also the apparent coincidence of two activities; linoleate oxidation and Chl bleaching.

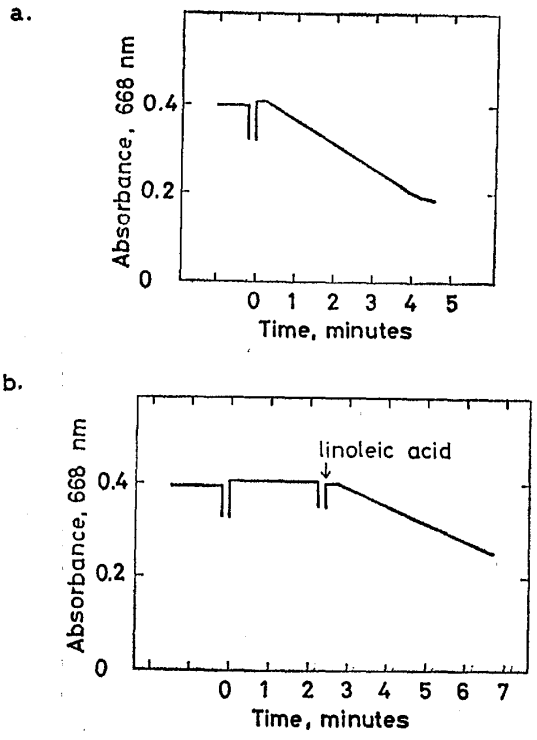

Fig. 1. Chlorophyll bleaching as evidenced by decrease in absorption at 668 nm. (a) Complete reaction mixture. (b) Reaction mixture containing linoleate hydroperoxide instead of linoleic acid. Chlorophyll bleaching was apparent after the addition of linoleic acid.

Purification of lipoxygenase

Lipoxygenase from wheat and potato was purified by the steps summarized in Tables 3 and 4, re-

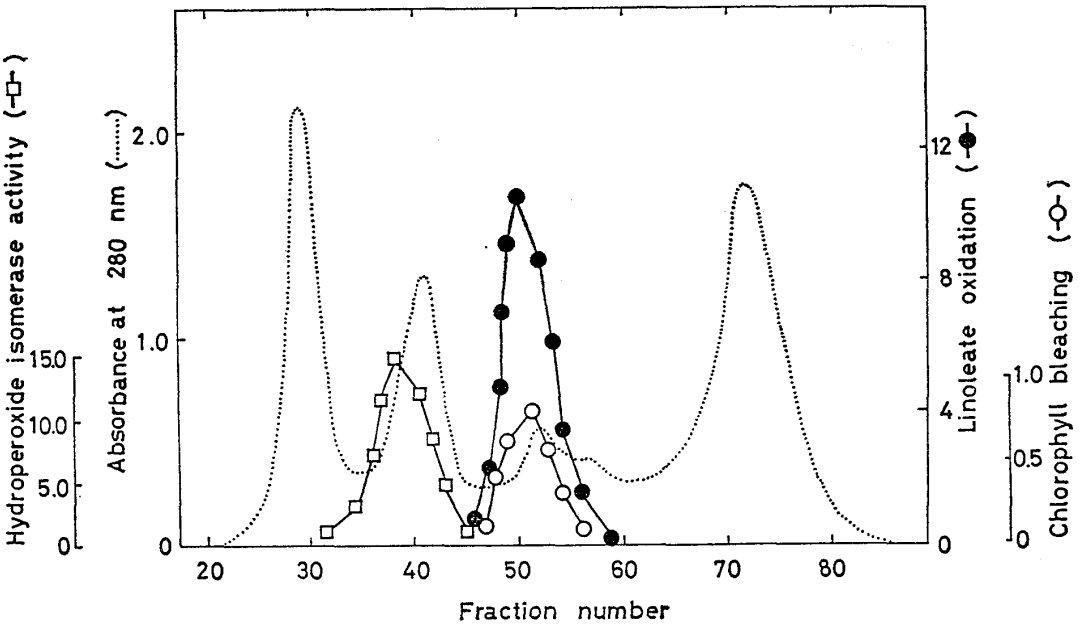

Fig. 2. Elution pattern of activities of hydroperoxide isomerase, linoleate oxidation and chlorophyll bleaching of wheat extract from Sephadex G-200. Fractions of 6 ml were collected and assayed.

spectively. Fractions having linoleate oxidizing activity always bleached Chl. On account of its unstable nature, only 6.9-fold purification was achieved for linoleate oxidizing activity of potato tuber enzyme. Neither 2-mercaptoethanol (1 to 10 mM) or reduced glutathione (1 mM) could stabilize the enzyme. The ratios of specific activities of linoleate oxidation to Chl bleaching were different in wheat and in potato and also at the different steps of purification; *i. e.*, 46 for crude wheat extract, and 59 for partially purified enzyme; 11 for crude potato extract, and 14 for partially purified enzyme. Increase in this ratio may suggest the loss of Chl bleaching factor during the purification process, or the inactivation of Chl bleaching activity which is essential for native lipoxygenase.

Since hemoproteins such as catalase, peroxidase, and cytochrome c are able to peroxidize lipids, involvement of these factors in the present Chl bleaching system was investigated. By gel-filtration of potato tuber preparation, catalase and peroxidase activities were found in peaks different from that of the Chl bleaching activity (Fig. 3). Moreover, at the final step of purification, no hematin was detected in lipoxygenase preparation by the method described by Hartree[25].

Effects of some compounds

Effects of various compounds on activities of linoleate oxidation and Chl bleaching were tested (Table 5). Both linoleate oxidation and Chl bleaching were inhibited by lipid antioxidants; α-tocopherol and

Table 3. Purification of wheat lipoxygenase

Purification step	Total protein (mg)	Total activity		Recovery (%)		Specific activity	
		Linoleate oxidation	Chlorophyll bleaching	Linoleate oxidation	Chlorophyll bleaching	Linoleate oxidation	Chlorophyll bleaching
Crude extract	463	3056	66.2	100	100	6.6	0.143
$(NH_4)_2SO_4$ ppt. 0-50% satn.	220	2552	42.2	84	64	11.6	0.192
DEAE-Sephadex	20	2200	20.6	72	31	110.0	1.03
Sephadex G-200	4.3	1116	19.0	37	29	259.5	4.41

Table 4. Purification of potato tuber lipoxygenase

Purification step	Total protein (mg)	Total activity		Recovery (%)		Specific activity	
		Linoleate oxidation	Chlorophyll bleaching	Linoleate oxidation	Chlorophyll bleaching	Linoleate oxidation	Chlorophyll bleaching
Crude extract	882	5627	525	100	100	6.38	0.595
$(NH_4)_2SO_4$ ppt. 20-50% satn.	506	5667	497	101	95	11.2	0.982
Sephadex G-150	196	5155	337	92	64	26.2	1.72
DEAE-Sephadex	57	2497	178	44	34	43.8	3.13

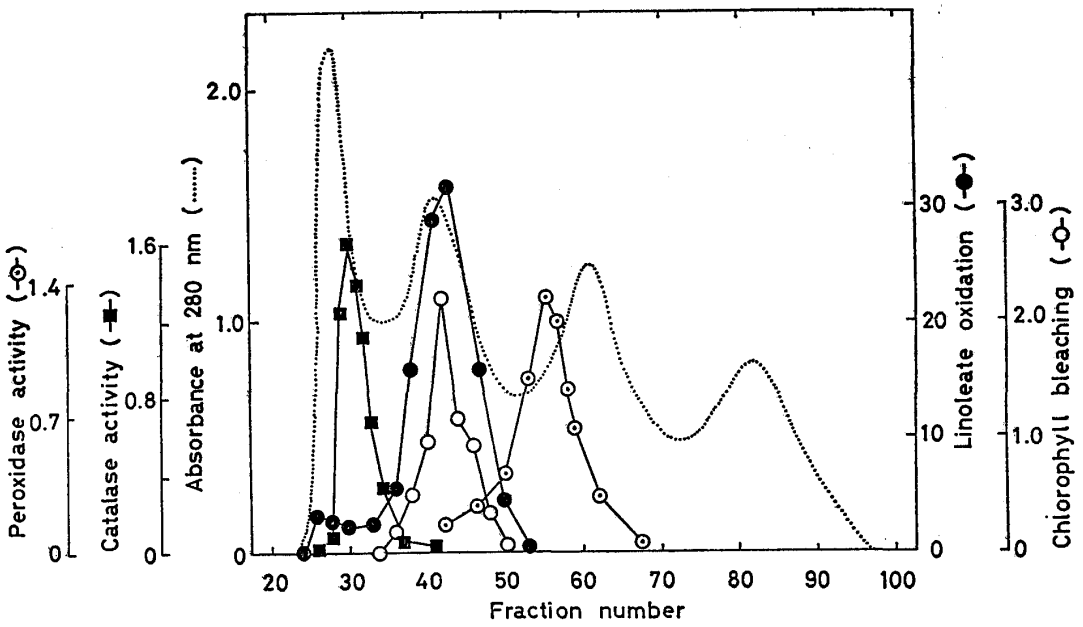

Fig. 3. Elution pattern of activities of linoleate oxidation, chlorophyll bleaching, peroxidase, and catalase of potato tuber extract from Sephadex G-200. Fractions of 6 ml were collected and assayed.

Table 5. Effects of various compounds on linoleate oxidation and chlorophyll bleaching by partially purified potato tuber lipoxygenase.

Compound tested	Concn. (M)	Activity* (% control)	
		Linoleate oxidation	Chlorophyll bleaching
Control		100	100
α-Tocopherol	1.5×10^{-3}	1	23
NDGA	1.5×10^{-4}	25	0
KCN	1.5×10^{-3}	108	100
NaN_3	1.5×10^{-3}	95	88
NaF	1.5×10^{-3}	72	92
Thiourea	1.5×10^{-3}	0	93
o-Phenanthroline	1.5×10^{-4}	0	94
DIECA	1.5×10^{-4}	65	12
	1.5×10^{-3}	0	0
Ascorbate	1.5×10^{-4}	72	20
p-Quinone	1.5×10^{-3}	0	31
$FeSO_4$	1.5×10^{-3}	75	114
$CuSO_4$	1.5×10^{-4}	75	67
$ZnSO_4$	1.5×10^{-3}	96	75
$MnSO_4$	1.5×10^{-3}	94	25
$Mg(C_2H_3O_2)_2$	1.5×10^{-3}	101	97

* Activity of control was taken as 100.

NDGA. Potato lipoxygenase was also inhibited by some metal-complexing agents, which is inconsistent in part with the facts previously reported that the enzyme from various sources was not inhibited by azide, CN^-, DIECA, F^-, pyrophosphate, and EDTA[26]. *o*-Phenanthroline and thiourea also inhibited linoleate oxidation, but not Chl bleaching. The exact nature of this lack of inhibition of Chl bleaching cannot be explained. These compounds might have reacted directly with Chl, since oxidation of fatty acid by lipoxygenase was apparently essential for Chl bleaching from other experimental data. Chl bleaching was inhibited by ascorbate and $MnSO_4$, but inhibition of linoleate oxidation was weaker, suggesting that these compounds might have uncoupled the bleaching of Chl with linoleate oxidation.

Reaction products of wheat lipoxygenase

Analysis of reaction products by thin-layer chromatography showed two spots corresponding to methyl 9- and methyl 13-hydroxy isomers (Fig. 4). The ratio of two isomers formed in the oxidation of linoleic acid by partially purified wheat enzyme at pH 6.5 was 88% of 13-isomer to 12% of 9-isomer. Soybean lipoxygenase is reported to produce mainly 13-isomer[27,28] and corn and potato tuber enzymes mainly 9-isomer[1,29]. From these observations and the results shown in Table 2, bleaching of Chl seems to occur independently of the kind of hydroperoxides produced.

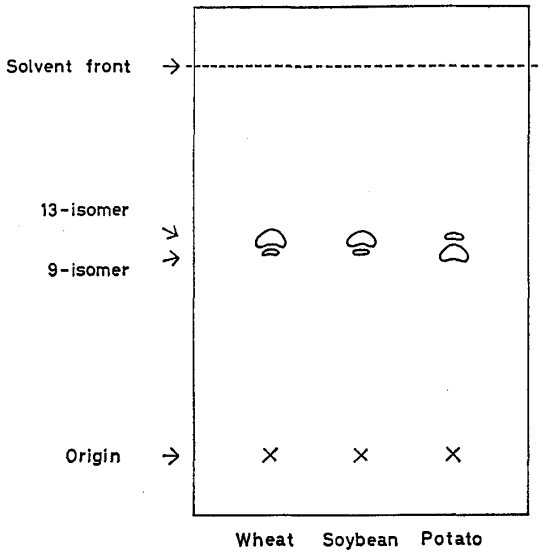

Fig. 4. Thin-layer chromatogram of reaction products of partially purified lipoxygenase. Methyl hydroxy derivatives of products were prepared and chromatographed on Silica Gel F_{254}.

Electrophoresis

Polyacrylamide gel zymograms of partially purified soybaen lipoxygenase are shown in Fig. 5 .The unstained gels were cut into 0.5 cm-thick segment and each segment was homogenized with reaction mixture (3 ml) in a mortar and pestle. After 2 min at room temperature, 3 ml of ether was added and the mixture was shaken vigorously. Then it was centrifuged and the absorption of the ether layer was measured at 234 nm (conjugated diene hydroperoxides) and 668 nm (Chl *a*). Other gels run at the same time were stained with Coomassie brilliant blue and *o*-dianisidine to detect protein and lipoxygenase activity, respectively. Part of the gel corresponding to 1.5-2.0 cm from the gel top was stained strongly with

these two staining dyes. The same part of the gel also showed the highest activities of linoleate oxidation and Chl bleaching.

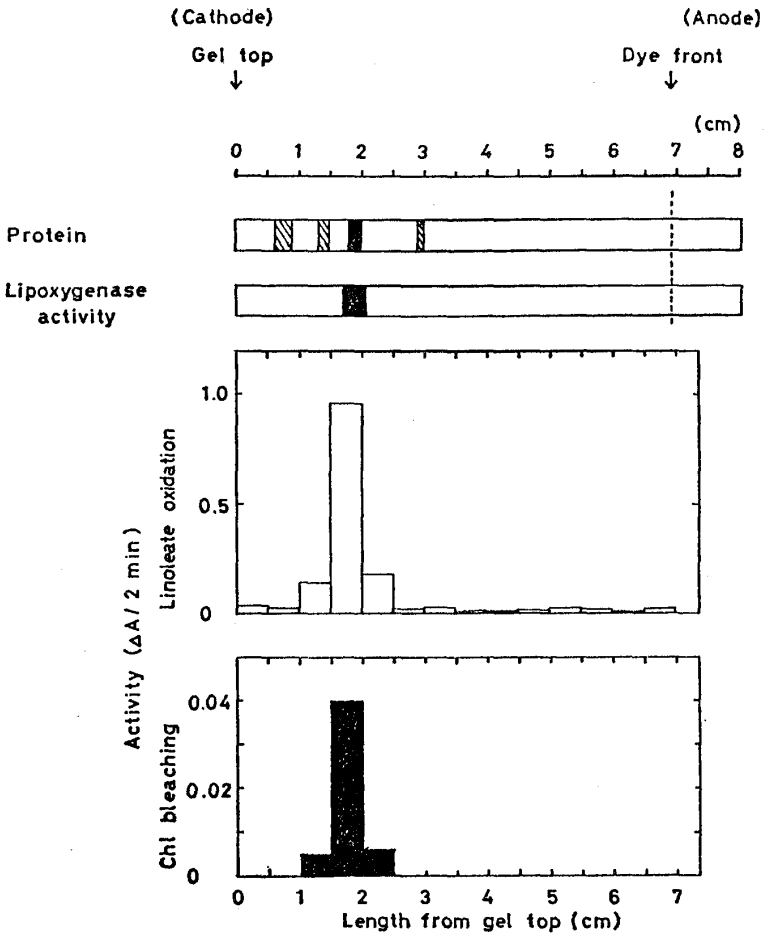

Fig. 5. Distribution of linoleate oxidizing and chlorophyll bleaching activities of partially purified soybean lipoxygenase after disk-gel electrophoresis. For details, see text.

Specificity against chlorophyllous compounds

Substrate specificity of Chl bleaching activity of partially purified potato lipoxygenase was tested. Table 6 shows that derivatives of

Table 6. Bleaching of some chlorophyllous compounds.

Derivative	Mg	Phytol chain	Bleaching*
Chlorophyll *a*	+	+	100
Chlorophyll *b*	+	+	99
Ethyl chlorophyllide *a*	+	−	118
Zn-Pheophytin *a*	−	+	56**
Pheophytin *a*	−	+	4
Ethyl pheophorbide *a*	−	−	36

* A relative amount of bleached pigment expressed in mole.
** Based on the absorption change, since the molar absorption coefficient is unknown.

Chl *a* with no Mg ion (pheophytin *a* and ethyl pheophorbide *a*) are not bleached so effectively as the derivative with Mg ion (ethyl chlorophyllide *a*). Absence of phytol chain seems to promote bleaching. Chl *b* was bleached as effectively as Chl *a*. Reaction product(s) of the chlorophylls in the present bleaching system has not been identified. As reported by Holden[8], the absorption of chlorophyll decreased greatly throughout the visible range as reaction proceeded. Neither new peaks nor the shift of peaks was observed, which suggests that Chl is rapidly converted into a colorless compound(s).

Time course of lipoxygenase activity and chlorophyll content of intact plants during growth and senescence

When wheat was water-cultured without the addition of nutrient, their 1st leaf began to loss Chl after 6 to 9 days of germination. Changes in lipoxygenase activity (linoleate oxidation and Chl bleaching) and Chl content for the initial 23 days were measured (Fig. 6).

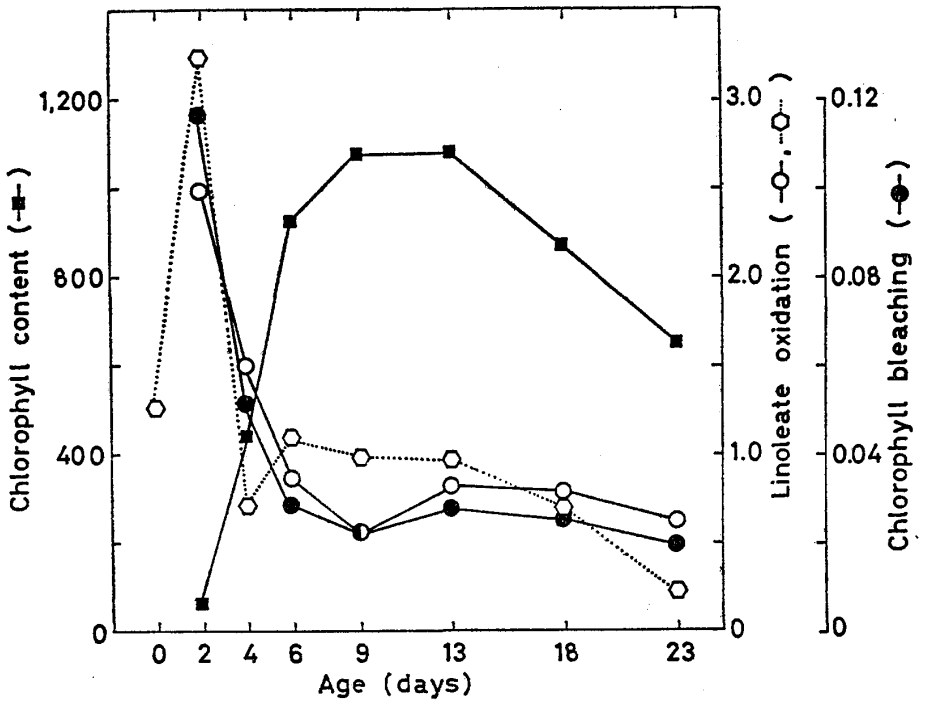

Fig. 6. Changes in chlorophyll content and lipoxygenase activity of wheat leaves during initial 23 days of growth. (-■-) Chlorophyll content of leaves; (···○···) linoleate oxidizing activity of dormant or remaining seeds; (-○-) linoleate oxidizing activity of leaves; and (-●-) chlorophyll bleaching activity of leaves.

Dormant seeds had rather low lipoxygenase activity. The activity increased greatly after 2 days of germination both in leaves and in remaining seeds followed by a sudden decline and subsequent period of gradual decrease. Increase in Chl content of leaves was great

during initial 4 days of 1st leaf formation. Chl content of the whole seedling increased gradually thereafter, probably due to the formation of 2nd leaf, and began to decline, until the 1st leaf became completely yellow. Lipoxygenase activity in 6-day-old wheat was relatively high in all the parts except in the mature part of 1st leaf (described as "Apical segment" in Table 7) which had the highest chlorophyll content. In 21-day-old plant, the activity was highest in developing 3rd leaf. Yellowing 1st and mature 2nd leaves had much lower activities than that of the 3rd leaf. Similar results were obtained with barley. From these results, lipoxygenase activity seems to be high in growing tissues and decline as they pass into the mature phase of development.

Table 7. Lipoxygenase activity and chlorophyll content of various parts of wheat.

Part	Chlorophyll content (μg/g fr. wt.)	Linoleate oxidation (ΔA/min/mg)
6-day-old		
Root	—	3.07
Remaining seed	—	3.24
Basal 2-cm segment	244	3.21
Segment 2-4.5 cm from the base containing growing point	822	3.06
Apical 3.5-cm segment	1,536	1.66
21-day-old		
Root	—	0.31
Remaining seed	—	0.71
First leaf	277	0.53
Second leaf	907	1.35
Third leaf	862	2.32

Five-day-old leaves of barley incubated on a filter paper moistened with one of the incubation media in a covered Petri dish. Incubation medium was (i) 10 mM phosphate buffer, pH 6.8, (ii) the same buffer containing 10^{-10} M kinetin, or (iii) the same buffer containing 10^{-5} M cycloheximide. Petri dishes containing samples were incubated in the dark at 27±1°C. Leaves incubated with buffer lost Chl rapidly and retained only 9% of the initial chlorophyll content after 96 hr of incubation. Lipoxygenase activity decline also. Kinetin and cycloheximide were reported to delay senescence of excised oat leaf segment[30]. Both kinetin and cycloheximide were effective for barley in delaying the loss of Chl, but the response of lipoxygenase activity to these compounds opposed the view that lipoxygenase-catalyzed Chl bleaching system is active in senescent leaves (Fig. 7).

It is, of course, not possible from the results described above to give any conclusion, except that at least lipoxygenase-unsaturated

fatty acid system does not play a predominant role in Chl degradation *in vivo*.

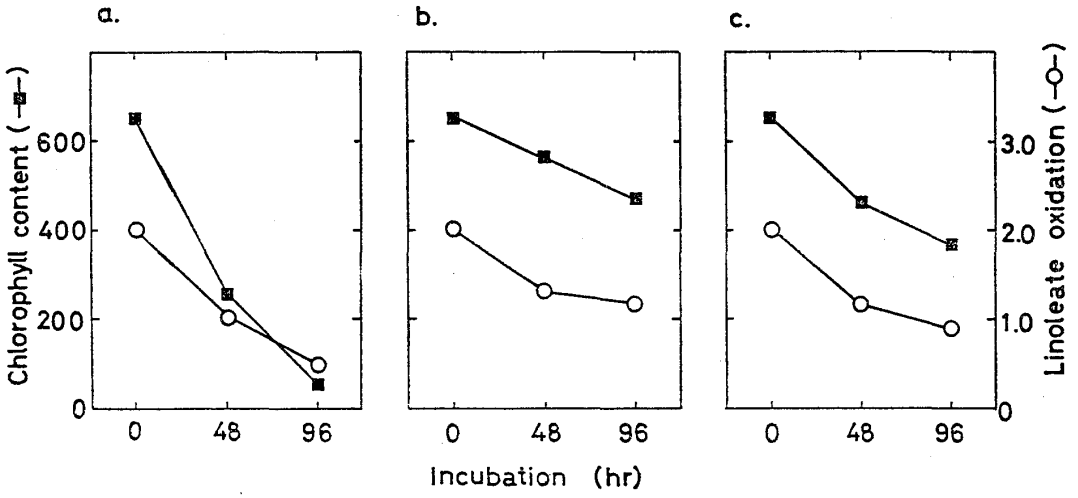

Fig. 7. Changes in chlorophyll content and lipoxygenase activity of excised barley leaves. Leaves were incubated with (a) 10 mM phosphate buffer, pH 6.8, (b) the same buffer containing 10^{-4}M kinetin, or (c) the same buffer containing 10^{-5}M cycloheximide.

Discussion

The *in vitro* bleaching of Chl in the presence of linoleic acid was catalyzed by extracts of various plant sources and lipoxygenase was shown to be necessary for this process. We have shown that the oxidation products of linoleate by lipoxygenase did not bleach Chl in the presence of hydroperoxide isomerase (Fig. 1), and that lipoxygenase free from hydroperoxide isomerase activity did bleach Chl during linoleate oxidation (Fig. 2), involvement of hydroperoxide isomerasc in the Chl bleaching process being doubtful. Zimmerman and Vick[9] reported briefly that lipoxygenase alone would not bleach Chl in the presence of linoleic acid, and that bleaching occurred only in the presence of linoleic acid, lipoxygenase and hydroperoxide isomerase. Inconsistence between their observation and our results might be ascribed to the contamination of lipoxygenase activity in the faction of hydroperoxide isomerase.

During the course of this study, several papers were published in this line of subjects: Orthoefer and Dugan[31] reported that Chl was not bleached with previously oxidized fatty acids. Weber *et al.*[32,33] observed that carotene was not oxidized by the linoleic acid hydroperoxides but showed that homogeneous preparation of pea lipoxygenase bleached carotene and Chl in the presence of linoleic acid and O_2. The oxidation of carotene was proposed to be due to radicals produced from linolcic acid. Weber *et al.*[33] also suggested that, in pea lipoxygenase, isoenzyme with a pH optimum of 6.5 oxidizes carotene

with linoleic acid as co-substrate, but isoenzyme with a pH optimum of 9.0 (Theorell enzyme) has a low carotene oxidizing activity. In this study, however, purified soybean lipoxygenase actively bleached Chl in the presence of linoleic acid at pH 6.5 but not at pH 8.8 which was optimum for linoleate oxidation by this enzyme. In contrast to soybean lipoxygenase, both crude and partially purified wheat enzyme oxidized linoleic acid only at acidic pH and not at pH 9.0. Their activity to bleach Chl were, however, rather low compared to soybean enzyme even at acid pH values; *i.e.*, the activity ratio of linoleate oxidation to Chl bleaching was higher in wheat lipoxygenase than in soybean. So, it cannot be concluded that only the isoenzyme with an acidic pH optimum bleaches Chl and that isoenzyme with an alkaline pH optimum does not.

Then how could the difference in the activity ratio of linoleate oxidation to Chl bleaching be explained? From the observation that the increase in the above activity ratio was apparent with the advance of purification, one possible explanation is that only the intact enzyme does bleach Chl during the oxidation of linoleic acid but the enzyme which lost a certain part of its component does not. Indeed, by the treatment with protease Chl bleaching activity of commercial soybean lipoxygenase was much more reduced than linoleate oxidizing activity was. Action of proteolytic enzymes during extraction and purification procedures may affect the Chl bleaching activity of lipoxygenase.

Another possible explanation is the contamination of inhibitory substances. Crude wheat extract prepared from the acetone powder of this plant contained a substance which was inhibitory for lipoxygenase activity. This substance could not be separated by $(NH_4)_2SO_4$ fractionation but effectively separated by gel-filtration on Sephadex-G-200. This substance was supposed to be glutenin, which might modify the reaction process of lipoxygenase, as suggested by Graveland[34]. In this study, crude enzyme was extracted from fresh wheat leaves, so the contamination of this inhibitory substance was negligible.

Since soybean lipoxygenase was not inhibited by compounds which chelate metals[26], it was generally approved that the enzyme does not contain any metals. Recently, however, several papers were published which suggested the presence of iron atom in soybean lipoxygenase[35-37]. In this study linoleate oxidizing aciivity of wheat and potato lipoxygenase was inhibited in some extent by chelating agants (Table 5), iron atom may be found in some different forms other than heme iron in enzyme protein.

Although much work has been done on lipoxygenase, its physiological function in higher plants is still obscure. Using dwarf pea seedlings, Anstis ahd Friend[38] suggested tnat lipoxygenase is not involved directly in photosynthesis or chloroplast development. In this study, changes in lipoxygenase activity and Chl content of leaves

during growth and senescence were measured and the results obtained failed to indicate a positive relationship between lipoxygenase activity and Chl loss in yellowing leaves. The fact that lipoxygenase activity was rather high in young tissues where chlorophyll was actively synthesized (Table 7) may suggest that lipoxygenase does not function in Chl bleaching reaction *in vivo*.

Throughout this study, no direct evidence for the participation of lipoxygenase-unsaturated fatty acid system in the degradation of Chl *in vivo* has been obtained. However, there are observations that complex lipid level of chloroplasts rapidly declined during leaf senescence and that the decline of linolenic acid content in chloroplast was in parallel with the decline of Chl content (M. Abe, personal communication). These facts suggest that there may be another lipid peroxidizing system which is coupled with Chl degradation. Furthermore, possibility remains that peroxides produchd in many kinds of reactions may serve as oxidizing agents for Chl; *i.e.*, hydrogen peroxide generated in glycolate oxidation bleached Chl *in vitro*. These must be left for future study.

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References

1) Galliard, T. and D.R. Phillips: Biochem. J., 124, 431 (1971).
2) Holden, M.: Phytochem., 9, 507 (1970).
3) Grossman, S., A. Ben Aziz, P. Budowski, I. Ascarelli, A. Gertler, Y. Birk and A. Bondi: Phytochem., 8, 2287 (1969).
4) Zimmerman, D.C. and B.A. Vick: Lipids, 8, 264 (1973).
5) Sumner, J.B. and R.J. Sumner: J. Biol. Chem., 134, 531 (1940).
6) Kies, M.W., J.L. Haining, E. Pistorius, D.H. Schroeder and B. Axelrod: Biochem. Biophys. Res. Commun., 36, 312 (1969).
7) Theorell, H., R.T. Holman and A. Akeson: Acta Chem. Scand., 1, 571 (1947).
8) Holden, M.: J. Sci. Fd. Agric., 16, 312 (1965).
9) Zimmerman, D.C. and B.A. Vick: Plant Physiol., 46, 445 (1970).
10) Vick, B.A. and D.C. Zimmerman: Proc. North Dakota Acad. Sci., 22, 29 (1968).
11) Draper, S.R.: Phytochem., 8, 1641 (1969).
12) Wolf, F.T., J.G. Coniglio and R.B. Bridges: In "Biochemistry of Chloroplasts" I. Edited by T.W. Goodwin. p. 187. Academic Press, 1966.
13) Hitchcok, C. and B.W. Nichols: In "Plant Lipid Biochemistry" p. 259. Academic Press, 1971.
14) McFeeters, R.F., C.O. Chichester and J.R. Whitaker: Plant Physiol., 47, 609 (1971).
15) Holt, A.S. and E.E. Jacobs: Amer. J. Bot., 41, 710 (1954).
16) Sherman, G., T.M. Korn and H. Linschitz: Photochem. Photobiol., 16, 499 (1972).
17) Surrey, K.: Plant Physiol., 39, 65 (1964).
18) Hackett, D.P.: In "Modern Methods of Plant Analysis" VII. Edited by K.

Paeck and M. V. Tracey. p. 685. Springer-Verlag, 1964.
19) Beers, R. F. and I. W. Sizer: J. Biol. Chem., **195**, 133 (1952).
20) Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. J. Randall: J. Biol. Chem., **193**, 265 (1951).
21) Arnon, D. I.: Plant Physiol., **24**, 1 (1949).
22) Davis, B. J.: Ann. N. Y. Acad. Sci., **121**, 404 (1964).
23) Weber, K. and M. Osborn: J. Biol. Chem., **244**, 4406 (1969).
24) Verhue, W. M. and A. Francke: Biochim. Biophys. Acta, **285**, 43 (1972).
25) Hartree, E. F.: In "Moderd Methods of Plant Analysis" IV. Edited by K. Paech and M. V. Tracey. p. 214. Springer-Verlag, 1955.
26) Tappel, A. L.: "The Enzyme" VIII. Edited by P. D. Boyer, H. Lardy and K. Myrback. p. 282. Academic Press, 1963.
27) Dolev, A., W. K. Rohwedder and H. J. Dutton: Lipids, **2**, 28 (1967).
28) Christopher, J. and B. Axelrod: Biochem. Biophys. Res. Commun., **44**, 731 (1971).
29) Gardner, H. W.: J. Lipid Res., **11**, 311 (1970).
30) Martin, C. and K. V. Thimann: Plant Physiol., **49**, 64 (1972).
31) Orthoefer, F. T. and L. R. Dugan, Jr.: *ibid.*, **24**, 357 (1973).
32) Weber, F., D. Arens and W. Grosch: Z. Lebensm. Unters. Forsch., **152**, 152 (1973).
33) Weber, F., G. Laskawy and W. Grosch: *ibid.*, **152**, 324 (1973).
34) Graveland, A.: J. Am. Oil Chem. Soc., **47**, 452 (1970).
35) Roza, H. and Francke, P.: Biochim. Biophys. Acta, **327**, 24 (1973).
36) Chan, H. W. S.: *ibid.*, **327**, 32 (1973).
37) Pistorius, E. K. and B. Axelrod: J. Biol. Chem., **249**, 3183 (1974).
38) Anstis, P. J. P. and J. Friend: Phytochem., **13**, 2709 (1974).