Chlorophyll Inhibition of Lipoxygenase in Growing Pea Plants

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A comparison of the lipoxygenases isolated from light-grown and etiolated pea plants indicated that they were identical with the same pH profile, electrophoretic mobility, and similar K_m's on linoleate. The decrease in lipoxygenase activity in light-grown pea plants was not due to water-soluble inhibitors. Added chlorophyll greatly inhibited the enzyme when isolated from light-grown or dark-grown plants. Supporting evidence was also obtained when it was found that chlorophyll content of growing plants increased with time in the same proportion as the decrease in lipoxygenase activity. When chlorophyll was removed, no inhibition of lipoxygenase activity could be detected. TLC of the products of linoleate oxidation gave one major product from the light-grown plant (R_f 0.41) and two from the etiolated plant (R_f 0.41 and 0.35). The product with the R_f of 0.41 has the typical UV spectrum of linoleate hydroperoxide with maximum absorbance at 234 nm. The product with R_f of 0.35 exhibited a pronounced peak at 285 nm characteristic of conjugated dienone chromophores.

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the hydroperoxidation of polyunsaturated fatty acids interrupted by *cis*-methylene (Axelrod, 1974; Eskin et al., 1977; Veldink et al., 1977). The enzyme has been extensively studied in a variety of plant and animal tissues. In spite of the attention lipoxygenase has received, its physiological role in plants has not been completely elucidated although many have been suggested (Axelrod, 1974; Shahin and Grossman, 1978; Galliard and Chan, 1980; Vick and Zimmerman, 1983). The activity of lipoxygenase in oxidizing polyunsaturated fatty acids is accompanied by the bleaching of pigments such as carotene and chlorophyll (Grosch et al., 1977; Pistorius and Axelrod, 1974; Ramadoss et al., 1978; Yoon and Klein, 1979; Holden, 1965; Imamura and Shimizu, 1974).

Since the study by Holden in 1965 on decoloration of chlorophyll during fatty acid oxidation by lipoxygenase in legume seeds, a few other studies both with crude extracts and purified enzymes have been undertaken in order to understand this cooxidation process (Zimmerman and Vick, 1970; Weber et al., 1973a,b; Imamura and Shimizu, 1974; Yoon and Klein, 1979; Reynolds and Klein, 1982). It was found that the oxidation of polyunsaturated fatty acids is inhibited by the cooxidized pigments (Yoon and Klein, 1979). The present study describes the part played by chlorophyll in the inhibition of lipoxygenase in the pea plant.

MATERIALS AND METHODS

Preparation of Plant Material. Pea plants (*Pisum sativum* L.) were grown in a greenhouse at 25 °C for 8–10 days in a loam–vermiculite mixture under controlled humidity. When etiolated plants were prepared, the seedlings were grown under the same conditions, but in addition they were covered with black cloths to avoid light penetration. Leaves were harvested for assay as described in the following sections.

Crude Homogenate Preparation. Three grams of pea leaves (without stems) was ground in 15 mL of 5 mM potassium phosphate buffer, pH 6.5, with a mortar and pestle. The homogenate obtained was centrifuged at 12000*g* for 10 min at 4 °C, and the supernatant was collected and assayed for lipoxygenase activity.

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When the homogenate was prepared for affinity chromatography experiments, 5 mM sodium acetate buffer (pH 5.0) was used instead of potassium phosphate buffer.

Enzyme Assay. Lipoxygenase activity with linoleate as substrate was assayed by two different techniques: (a) oxygen absorption (linoleate 7.5×10^{-3} M), measured polarographically according to Grossman and Zakut (1979), and (b) spectrophotometric measurement (linoleate 7.5×10^{-4} M) of conjugated diene formation according to Ben-Aziz et al. (1970).

Protein Determination. Protein concentration was determined by the method of Bradford (1976).

Purification of Enzymes by Affinity Chromatography. The affinity chromatography on linoleyl(aminoethyl)-Sepharose was performed according to the method of Grossman et al. (1972), which was slightly modified by using Sepharose 4B (Pharmacia, Uppsala, Sweden) instead of agarose. Three milliliters of the homogenate supernatant were applied to a linoleyl(aminoethyl)–Sepharose column (6 × 1 cm) equilibrated with 5 mM sodium acetate buffer, pH 5.0, at 4 °C. After 20 min of incubation, the column was washed with the equilibrating buffer until the absorption at 280 nm was reduced to zero. The active enzyme was eluted with 200 mM sodium acetate buffer at pH 5.0.

Isolation of Products. The products of linoleic acid oxidation by the crude extract or purified enzyme were prepared by adding 5 mg of purified enzyme, or 5 mL of crude homogenate, to 30 mL of 7.5 mM linoleate solution containing 0.25% Tween-20 in 200 mM sodium phoshate buffer, pH 6.5 (Ben-Aziz et al., 1970).

After incubation for 30 min (with gentle stirring) at room temperature, the mixture was acidified to pH 3 and extracted with diethyl ether, and the enzyme reaction products were chromatographed on silica gel plates (DC-Karten SIF, Riedel-De Haen Aktiengesellschaft Seeize-Hanover) with petroleum ether–diethyl ether–acetic acid (60:40:1 v/v) (Zimmerman and Vick, 1973) as the solvent. Products were visualized under ultraviolet light and eluted from the silica gel with ethyl ether. The absorption spectra of the products in 100 mM borate buffer, pH 9, were measured with the aid of a double cuvette, with a Gilford spectrophotometer, Model 250.

Estimation of Chlorophyll. The amount of chlorophyll in the leaves was determined spectrophotometrically from the absorbance at 663 and 645 nm according to the method of Anderson and Brenner (1977).

Endogenous Antioxidant Levels. The endogenous antioxidant content in leaves was measured by following

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Table I. Lipoxygenase Activity of the Crude and Purified Extracts of Etiolated and Light-Grown Pea Plants[a]

	specific activity					
	diene formation, OD_{234} (mg of protein)$^{-1}$ min^{-1}			oxygen absorption, μL of O_2 (mg of protein)$^{-1}$ min^{-1}		
sample	crude	purified	enrichment	crude	purified	enrichment
light	2.99	15.68	5.24	2.92	88.15	30.18
dark	9.01	20.00	2.22	43.26	93.80	2.16

[a] Enzyme samples were prepared and assayed as described under Materials and Methods.

the ability of leaf extract to inhibit linoleate oxidation by commercial soybean lipoxygenase.

The extracts were prepared as follows: The supernatant of the homogenate (prepared with water as described above) was boiled for 30 min and centrifuged for 10 min at 12000*g*. The supernatant was evaporated to dryness by boiling and then dissolved in water to give a final concentration of solids of 2 mg/mL. Preliminary studies showed that dialysis or lyophilization of the supernatant provided similar results but the procedures required considerably more time than evaporation.

Samples of this solution at various concentrations were added to a mixture of 2.5 mL of linoleate (7.5×10^{-3} M) and 0.5 mL of commercial lipoxygenase (300 mg/mL) solutions. Oxygen uptake was followed polarographically by using a YSI biological oxygen monitor.

The degree of inhibition by antioxidants was determined by comparing the oxygen uptake with and without the leaf extracts.

Effect of Chlorophyll on Lipoxygenase Activity. One milligram of chlorophyll *a* was dissolved in 10 mL of acetone containing 0.035 mL of Tween-80. One milliliter of this solution was evaporated to dryness under a stream of N_2 and diluted with 2 mL of 0.2 M sodium phosphate buffer, pH 6.5. Different amounts of this solution were added to reaction mixtures containing linoleic acid (7.5×10^{-4} M) with various amounts of enzyme (pea lipoxygenase) and absorbance at 234 nm was measured. The control solutions were prepared similarly without chlorophyll.

The effect of chlorophyll *a* on lipoxygenase activity of pea plant extract was measured as described above with reference to endogenous antioxidants.

RESULTS AND DISCUSSION

Lipoxygenase Activity. Lipoxygenase activity of crude and purified extracts of etiolated and light-grown pea plants is shown in Table I. The specific activity of lipoxygenase in the crude extracts of etiolated plants was much higher than in light-grown plants. Moreover, this difference between dark- and light-grown plants was much more pronounced (about 15-fold) when lipoxygenase activity was determined by oxygen absorption as compared to the activity measured by conjugated diene production (3-fold). The corresponding enzymes purified by affinity chromatography, however, exhibited similar specific activities when activity was measured by either method. In addition, it was found that the protein level in both dark- and light-grown peas lipoxygenase active fractions was almost the same (Figure 1). These findings indicate that the level of lipoxygenase activity in both etiolated and light-grown plants is similar and this level cannot account for the difference in linoleate oxidation activities in the crude extracts of both types (Table I). In addition, both purified enzymes had the same pH profile with an optimum at pH 6.5, the same electrophoretic mobility, and

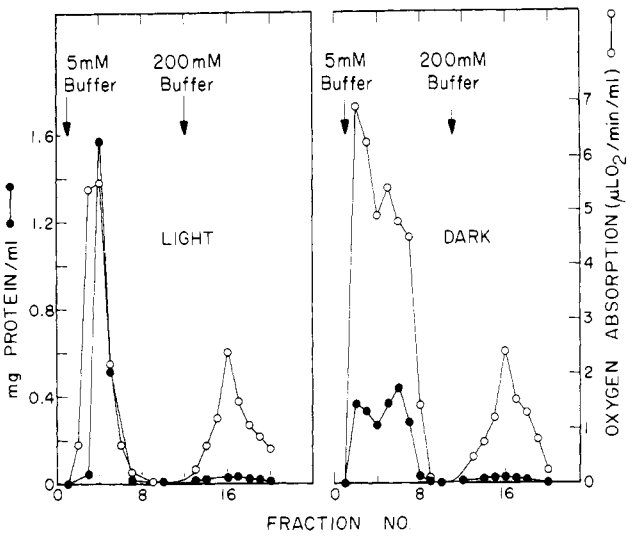

Figure 1. Affinity chromatography profiles of lipoxygenase from etiolated and light-grown pea plants on linoleyl(aminoethyl)-Sepharose. 3 mL of homogenate supernatant (etiolated, 6.9 mg; light, 8.8 mg) was applied to each column equilibrated with 5 mM sodium acetate buffer, pH 5.9. Elution of active fraction was performed with 200 mM of the same buffer. Lipoxygenase activity was assayed polarographically at pH 6.5 on linoleate and protein was estimated with Coomassie blue.

Table II. Effect of Water-Soluble Inhibitors from Etiolated and Light-Grown Pea Leaves on Lipoxygenase Activity[a]

	inhibitor source			
	light-grown pea		etiolated pea	
inhibitor extract, μL	oxygen absorption, μL of O_2/min	inhibition, %	oxygen absorption, μL of O_2/min	inhibition, %
0	2.18	0	2.18	0
50	1.95	10.3	1.80	17.2
100	1.28	41.4	1.35	37.9
200	0.75	65.5	0.75	65.5
300	0.30	86.2	0.30	86.2

[a] Inhibitors were prepared from homogenates of pea leaves as described under Materials and Methods, and their inhibitory effect on linoleate oxidation induced by soybean lipoxygenase at pH 6.5 was followed polarographically.

similar K_m's on linoleate (4.2×10^{-4} M).

The inhibition of lipoxygenase activities, as measured by oxygen absorption and conjugated diene formation, can be attributed to other factors present in the light-grown extract. The possible involvement of two factors—endogenous inhibitors and chlorophyll—was studied.

It has been reported in the literature that water-soluble antioxidants or inhibitors are widely distributed in plants and are very effective in the inhibition of lipoxygenase and other related lipid peroxidizing factors (Pinsky et al., 1971; Rhee et al., 1979).

In order to check this possibility, water-soluble inhibitors were extracted from etiolated and light-grown pea leaves and their effect on lipoxygenase activity was followed (Table II). It can be seen from the data presented in Table II that the inhibitory effect of these compounds on the activity of soybean lipoxygenase is very similar. Oxygen uptake decreased to less than 15% of the initial levels, with inhibitors from both light- and dark-grown leaves. This discovery rules out the possibility that the low linoleate oxidation in light-grown peas, as compared to etiolated ones, is due to the presence of these inhibitors or

Table III. Inhibition of Lipoxygenase Activity of Etiolated Pea Plants by Chlorophyll a[a]

enzyme, μg	chlorophyll concn, M	oxygen absorption, μL of O_2/min	inhibition, %
200	0	13.20	
200	5.4×10^{-6}	9.60	27.2
100	0	7.80	
100	5.4×10^{-6}	3.90	50.0
20	0	1.65	
20	1.8×10^{-6}	0.75	54.3
20	5.4×10^{-6}	0.60	63.6

[a] Chlorophyll solutions prepared as described under Materials and Methods were added to extracts of etiolated pea leaves, and oxygen absorption on linoleate at pH 6.5 was polarographically followed.

Table IV. Effect of Chlorophyll a on Lipoxygenase from Light-Grown Pea Leaves Purified by Affinity Chromatography[a]

chlorophyll a, M	inhibition of lipoxygenase activity, %
1.8×10^{-7}	30.0
3.6×10^{-7}	38.0
5.4×10^{-7}	45.2
9.0×10^{-7}	61.9
1.3×10^{-6}	69.0
1.8×10^{-6}	76.1
3.6×10^{-6}	92.8

[a] Chlorophyll a solutions prepared as described under Materials and Methods were added to samples of purified enzyme (80 μg/mL), and absorbance at 234 nm on linoleate at pH 6.5 was spectrophotometrically followed.

antioxidants in the light-grown leaves or their absence in dark-grown leaves. However, the relative concentrations of the inhibitor in the plant material itself was not determined.

These results led us to consider the second possibility: the effect of chlorophyll. One of the outstanding differences between etiolated and light-grown plants is the high level of chlorophyll in the light-grown plants as compared to the etiolated ones. The increase in lipoxygenase activity noted in light-grown pea lipoxygenase after affinity chromatography (Table I) might be attributed to the removal of the chlorophyll. As can be seen from Table III, chlorophyll added in concentrations similar to that found in the light-grown leaves greatly inhibited the lipoxygenase activity of extracts from etiolated plants. A similar effect was found when the effect of chlorophyll on activity of the purified lipoxygenase from light-grown peas was measured (Table IV).

This inhibitory effect of chlorophyll on linoleate peroxidation could be inferred from the experiment in which the changes in chlorophyll concentration and lipoxygenase activity were related in peas grown in the dark and in daylight (Figure 2). It is clearly seen that transferring the pea plants from dark to light causes an increase in chlorophyll concentration, and there is a decrease in lipoxygenase activity as a function of length of time illuminated.

Products of Oxidation. The differences in the relative diene formation and oxygen absorption activity of crude extracts from light-grown and etiolated peas (2.99 to 9.01, conjugated dienes, and 2.92 to 43.26, oxygen absorption) (Table I) led us to analyze the linoleate oxidation products of both light- and dark-grown peas. The TLC pattern indicates that with lipoxygenase from light-grown plants

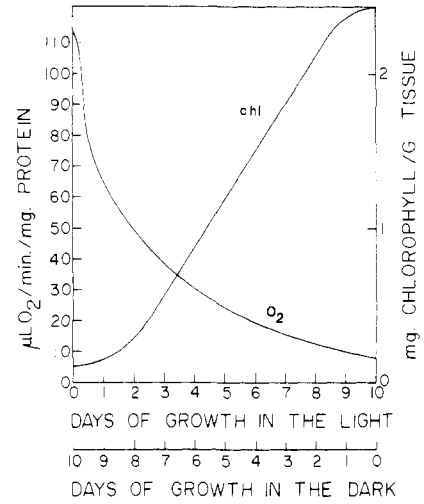

Figure 2. Changes in chlorophyll concentration and lipoxygenase activity as a function of the light received. The total growth period of each plant was 10 days in each case. There were 10 groups of plants with treatment ranging from total darkness to 10 days in light. Each group of plants was assayed for lipoxygenase activity and amount of chlorophyll.

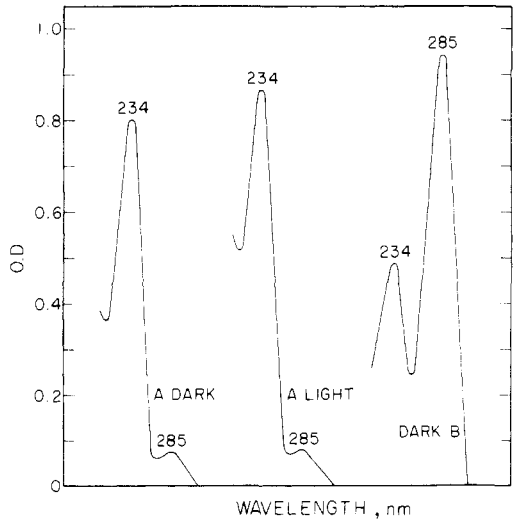

Figure 3. UV spectra of reaction products A and B isolated by TLC from pea lipoxygenases of light-grown and etiolated plants. Activity of lipoxygenase was measured on linoleate.

there is one major product (A, R_f 0.41), and the dark-grown oxidation products consisted of two oxidized components (A, R_f 0.41; B, R_f 0.35).

The UV spectra of both components A and B are shown in Figure 3. Compound A has the typical UV spectrum of linoleate hydroperoxide with a maximum absorbance at 234 nm. On the other hand, compound B exhibits a pronounced peak at 285 nm, characteristic of conjugated dienone chromophores. The UV spectra (Figure 3) indicate differences between the products derived from the lipoxygenase in light- and dark-grown peas. This observation may explain the relatively low activity of lipoxygenase from the crude extracts of dark-grown peas when measured by diene formation, since there is a possibility that two enzyme systems are present in the etiolated peas: lipoxygenase, which oxidizes the linoleic acid to linoleate hydroperoxide, and hydroperoxide isomerase, which converts the linoleate hydroperoxide to keto hydroxy compounds (Galliard and Chan, 1980). To establish this assumption, future research will be directed toward isolation and identification of the products separated by TLC.

The data presented in this study concerning the high level of lipoxygenase activity in etiolated plants compared

to light grown are in agreement with some other reports (Oelze-Karow et al., 1970, 1976). However, they contradict the opposite findings of Douillard and Bergeron (1978) in young wheat leaves where high lipoxygenase activity was noted in light-grown plants and low enzyme activity in etiolated plants. Zimmerman and Vick (1970) also reported that in mung bean seedlings the lipoxygenase activity in light- and dark-grown seedlings is similar. However, all these reports, except our present study, refer to the activity of lipoxygenase in crude extracts, and no attempt was made to isolate and purify the lipoxygenase activity. It is possible that in the crude extracts, in addition to chlorophyll, other factors such as endogenous antioxidants or inhibitors play an important role in the determination of lipoxygenase activity.

Since the specific activity of the lipoxygenase after purification in light- and dark-grown plants is comparable, and since the involvement of endogenous inhibitors or antioxidants was essentially ruled out (Table II), it can be concluded that in an in vitro system chlorophyll is an inhibitor. Therefore, chlorophyll could play an important role in the inhibition of lipoxygenase activity in light-grown leaves. These findings cast doubts on the assumption presented by Mohr (1972) that the increased activity of lipoxygenase in mustard seedlings grown under dark conditions represents synthesis of the enzyme. Mohr refers to the changes in activity when plants were changed from dark to light condition or vice versa as a demonstration of the rapidity of the threshold response in inhibition or resumption of lipoxygenase synthesis. Since all work was done with crude extracts, it is possible that the presence or absence of the chlorophyll in the extracts plays an important role in determining lipoxygenase activity, perhaps even to a greater extent than de novo synthesis of the enzyme.

Registry No. Lipoxygenase, 9029-60-1; chlorophyll *a*, 479-61-8; linoleate hydroperoxide, 78780-30-0.

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Enzymes of Glutamine and Asparagine Metabolism in Developing Wheat Grains

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Total protein, glutamate dehydrogenase, and aspartate pyruvate transaminase activities per grain increased during wheat grain development. Glutamate synthase and glutamate-pyruvate transaminase activities increased mainly during the early stages. Asparagine synthetase and glutamate-oxaloacetate transaminase activities declined at maturity. Activity of glutamine synthetase followed a decreasing pattern. Specific activities as well as activities per unit dry weight decreased at maturity. Compared to C-306 (low-protein variety), grains of Shera (high-protein variety) had higher activities of glutamate dehydrogenase, glutamate synthase, glutamate-pyruvate transaminase, and aspartate-pyruvate transaminase during later stages of grain development. It is suggested that in developing wheat grains, both the glutamate synthase cycle and glutamate dehydrogenase pathway may be operative in ammonia assimilation, the former predominating during early stages and the later playing a more active role during the later stages.

Protein malnutrition in developing countries is mainly due to the poor quality and quantity of protein in cereals, particularly wheat, which constitutes a major proportion of the staple diet. Any improvement in protein quality and quantity in cereals requires the basic understanding of the processes involved in protein accumulation in developing grains and a comparative study of these processes in a high- and a low-protein cultivar. The availability of amino acids at the site of protein synthesis in developing grain involves metabolism of amino acids and amides, particu-

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