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LIPOXYGENASE IN DARK-GROWN *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; dwarf pea; enzyme purification; lipid oxidation; lipoxygenase; isoenzymes.

Abstract—Lipid oxidizing activity has been detected in acetone powders from both dark- and light-grown dwarf pea seedlings. This activity has been shown by several methods to be due to lipoxygenase. The enzyme from dark-grown seedlings has been purified 5·7-fold by ammonium sulphate precipitation and gel filtration. CM-cellulose chromatography of the purified enzyme yielded four active fractions. The properties of the four lipoxygenase isoenzymes are described.

INTRODUCTION

THE ENZYME lipoxygenase, which catalyses the peroxidation of *cis-cis*-1,4-pentadiene structures, has been extensively studied in seeds of legumes[1] and other storage tissues.[2] Lipid peroxidation is often coupled to chlorophyll or carotenoid destruction. Not all such activity is due to lipoxygenase however, as most haem proteins will also perform carotene destruction coupled to linoleic acid peroxidation.[3–5] Although carotene destruction by leaf extracts has been studied the occurrence of lipoxygenase in leaf and stem tissue has been a controversial subject,[6] especially as Blain *et al.* have claimed that most fatty acid oxidizing systems in leaves are haematin compounds.[7,8]

Xanthoxin,[9,10] a potent, naturally occurring plant growth inhibitor, has been shown to be among the products of the action of soybean lipoxygenase on violaxanthin *in vitro*.[11] During a study of factors regulating the production of xanthoxin in red light treated pea seedlings, acetone powders which contained lipoxygenase activity were obtained. The properties of these powders and the partial purification and separation into four active fractions of lipoxygenase from etiolated pea shoots are described below.

[1] TAPPEL, A. L. (1961) *Autoxidation and Antioxidants* (LUNDBERG, W. O., ed.), Vol. I, p. 325, Interscience, New York.
[2] GALLIARD, T. and PHILLIPS, D. R. (1971) *Biochem. J.* **124,** 431.
[3] DICKS, J. W. and FRIEND, J. (1967) *Phytochemistry* **6,** 1193.
[4] DICKS, J. W. and FRIEND, J. (1968) *Phytochemistry* **7,** 1933.
[5] BEN-AZIZ, A., GROSSMAN, S., BUDOWSKI, P. and ASCARELLI, I. (1971) *Phytochemistry* **10,** 1823.
[6] HOLDEN, M. (1970) *Phytochemistry* **9,** 507.
[7] BLAIN, J. A., PATTERSON, J. D. E. and PEARCE, M. (1968) *J. Sci. Food Agric.* **19,** 713.
[8] BLAIN, J. A. (1970) *J. Sci. Food Agric.* **21,** 35.
[9] TAYLOR, H. F. and BURDEN, R. S. (1970) *Phytochemistry* **9,** 2217.
[10] TAYLOR, H. F. and BURDEN, R. S. (1970) *Nature* **227,** 302.
[11] FIRN, R. D. and FRIEND, J. (1972) *Planta* **103,** 263.

RESULTS

Properties of crude extract

Acetone powders prepared from 10-day-old dwarf pea seedlings solubilized to give a linoleate-oxidizing activity of 67·5 μmol oxygen consumed/min/g powder. Similar powders prepared from light grown seedlings have an activity of 44·7 μmol oxygen consumed/min/g powder. This activity was equivalent to 39 and 26 μg respectively of commercially available soybean lipoxygenase. The products of linoleate-oxidizing activity of both dark- and light-grown seedlings had absorption spectra with a peak at 234 nm, indicating the presence of a conjugated diene. The main product in both cases had the same R_f in petrol. (b.p. 60–80)–Et_2O–AcOH(70:30:1) as the products of soybean lipoxygenase. The specific activities of dark- and light-grown seedling extracts were 57 and 44% respectively of that of soybean lipoxygenase as measured by product formation. Boiled extracts showed no activity. Such linoleate-oxidizing activity could be due either to a lipoxygenase-type enzyme or to a haem-containing enzyme.[1] Further experiments were undertaken to try to assign the activity to one or other of these classes. Solubilized acetone powders from light-grown seedlings showed a linear relationship between linoleate-oxidizing activity and extract concentration and an optimum pH of 6·2. In the same system cytochrome-*c* had a maximum activity at pH 5·5 but was inactive at pH 6·2. In fact, in the assay system as described, peroxidase had no detectable linoleate-oxidizing ability and cytochrome-*c* had a maximum activity of 10 U/mg protein even at high concentrations. In case these concentrations were inhibitory[1,2,13] much lower cytochrome-*c* concentrations were used and

TABLE 1. COMPARISON OF PROPERTIES OF CRUDE PEA EXTRACT, SOYBEAN LIPOXYGENASE AND HAEM PROTEINS

Treatment	Effect of oxidizing activity of: Pea enzyme	Soybean lipoxygenase	Haem proteins
Boiling enzyme	Inhibits	Inhibits[6]	Does not inhibit[6]
Oleate as substrate	No reaction	No reaction[1]	Oxidizes[1]
EDTA	No effect	No effect[1]	No effect[3]
Cyanide	No effect	No effect[1]	Inhibits[3]
Kinetics	$V \propto (E)$	$V \propto (E)^1$	$V \propto (E)^{1/2}$

V = reaction rate; (E) = enzyme concentration.

shown to have an activity of less than 10 U/mg protein. Solubilized acetone powders from dark-grown seedlings had the following properties: (a) heating the extract to boiling inhibited the linoleate-oxidizing activity by 94%, (b) the extract was able to oxidize the salts of linoleic and linolenic acids to the same extent but had no effect on ammonium oleate, (c) the extract was not inhibited by 1 mM EDTA, (d) provided the final pH could be kept low, the extract was not inhibited by 10 mM KCN. All these properties together with the kinetic behaviour are similar to those of previously reported lipoxygenases and conflict with those of haem-containing proteins[1,2,6] (Table 1).

[12] BANKS, A., EDDIE, E. and SMITH, J. G. M. (1961) *Nature* **190**, 908.
[13] LEWIS, S. E. and WILLS, E. D. (1963) *Biochem. Biophys. Acta* **70**, 336.

Purification of crude extract

Ten-day-old etiolated dwarf pea seedlings (143 g) were homogenized in cold acetone to give 5·47 g dry acetone powder. This dissolved in 0·05 M phosphate buffer (pH 7) to give 905 mg protein that contained high lipoxygenase and peroxidase activites (Table 2). The protein fraction precipitating at 30–50% $(NH_4)_2SO_4$ saturation was enriched in lipoxygenase activity but had lost over 90% of its original peroxidase activity. (The catalase activity of the extract was originally low and was virtually eliminated by ammonium sulphate precipitation.) Gel filtration (Fig. 1), showed that peroxidase and lipoxygenase activity ran together just behind the high MW protein that was first eluted. After further precipitation and dialysis the specific activity of the lipoxygenase was considerably increased with little loss of total activity (Table 1).

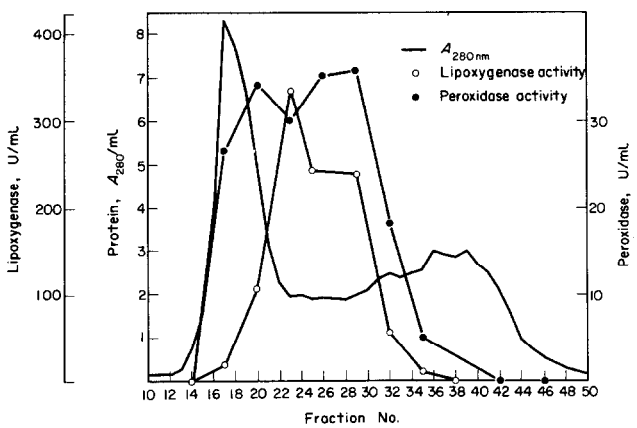

FIG. 1. GEL CHROMATOGRAPHY OF *Pisum sativum* SEEDLING LIPOXYGENASE.
Column: Sephadex G200 (2·7 × 60 cm) in 0·05 M phosphate (pH 7); Sample: 50% satd $(NH_4)_2SO_4$ precipitated enzyme.

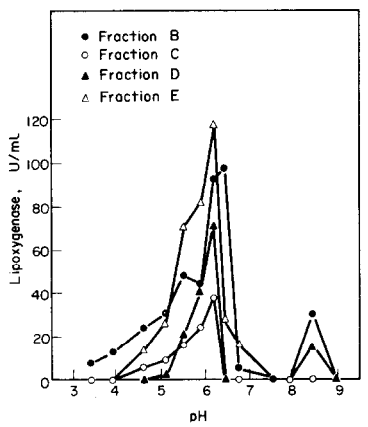

FIG. 2. EFFECT OF pH ON THE LIPOXYGENASE ACTIVITY OF FRACTIONS b–e ELUTED FROM CM-CELLULOSE.
Buffers: pH 3–8 acetate; pH 8–9 Tris HCl.

Five fractions were obtained on CM-cellulose, which corresponded to those obtained from alfalfa leaves by Grossman *et al.*[14] Fraction A, eluted with the starting buffer, con-

TABLE 2. LIPOXYGENASE AND PEROXIDASE LEVELS AT VARIOUS STAGES IN THE PURIFICATION OF ACETONE POWDERS FROM ETIOLATED PEA SEEDLINGS

		Lipoxygenase				Peroxidase			
Purification stage	Protein (mg)	Sp. act. (U/mg protein)	(%)	Total activity (units)	(%)	Sp. act. (U/mg protein)	(%)	Total activity (units)	(%)
Solubilized powder	905	126	100	114000	100	87	100	78900	100
30%$(NH_4)_2SO_4$ precipitate	144	9	7	1340	1	14	16	1980	3
50%$(NH_4)_2SO_4$ precipitate	300	197	156	59100	52	17	20	5200	7
G200, fractions 21–30	66	378	300	25000	22	25	29	2790	4
50%$(NH_4)_2SO_4$ precipitate dialysed	28	714	566	20200	18	45	52	1270	2

[14] GROSSMAN, S., BEN-AZIZ, A., BUDOWSKI, P., ASCARELLI, I., GERTLER, A., BIRK, Y. and BONDI, A. (1969) *Phytochemistry* **8**, 2287.

TABLE 3. LIPOXYGENASE ACTIVITY OF FRACTIONS ELUTED FROM CM-CELLULOSE

Fraction	Eluted with	Protein (mg)	(%)	Total activity (units)	(%)	Sp. act (U/mg protein)	Purification
A	0·005 M acetate	4·79	37	0	0	0	0
B	0·05 M acetate	2·16	17	2020	22	937	7·4
C	0·10 M acetate	2·42	19	1480	16	611	4·9
D	0·15 M acetate	0·99	8	2635	29	2660	21·1
E	0·1 M Na_2HPO_4	2·41	19	2965	33	1230	9·8
	Total	12·77	100	9100	100	1140	9·0

The acetate buffer was at pH 5·7. Purification values are compared with solubilized powder as 1·0.

tained the largest amount of protein. It had a very low peroxidase activity and no lipoxygenase activity. Fractions B, D and E were further enriched in lipoxygenase whereas the specific activity of fraction C was slightly decreased (Table 3). Peroxidase activity was only found in fractions A and E. Fraction E contained 99·4% of this activity, purified 3·8-fold with respect to the original extract.

Properties of active fractions

Each of the four linoleate-oxidizing fractions B–E was tested to discover the nature of this activity.

Optimum pH. The pH-activity profiles of the four fractions were similar but had important differences. Fractions C–E had their optimum pH at 6·2, whereas the optimum pH of fraction B was 6·45. To the alkaline side of these optima activity fell off very rapidly indeed. At pH 7·9, where none of the fractions were active, added soybeam lipoxygenase caused a rapid oxygen uptake. Fractions B and E had a shoulder in their pH-activity curves at pH 5·5 and fractions B and D showed a further small peak of activity at pH 8·45 in Tris buffer (Fig. 2).

TABLE 4. INFLUENCE OF DIFFERENT UNSATURATED FATTY ACIDS ON LIPOXYGENASE ACTIVITY OF FRACTIONS ELUTED FROM CM-CELLULOSE

Fraction	Rates of oxidation of different fatty acids		
	Linoleic	Linolenic	Oleic
B	100	89	0
C	100	122	0
D	100	149	25
E	100	181	0

Results expressed as % activity compared to linoleic acid.

All fatty acids were present as their ammonium salts at a final concentration of 1·2 mM and all fractions were assayed at pH 6·2.

Substrate specificity. All fractions oxidized linolenate very readily but only fraction D had any activity towards ammonium oleate (Table 4).

Effect of EDTA and cyanide. EDTA (0·1 mM) caused a slight stimulation of the activities of fractions B and E and inhibited fractions C and D. Because of its considerable effect on

pH and the alkali-sensitivity of the fractions, cyanide (10 mM) was tested at pH 5·5. Its greatest effect was on fraction C (Table 5).

TABLE 5. EFFECT OF CYANIDE AND EDTA ON THE LINOLEATE-OXIDIZING ACTIVITIES OF FRACTIONS ELUTED FROM CM-CELLULOSE

Fraction	% Stimulation (+) or inhibition (−)	
	Cyanide	EDTA
B	−18	+20
C	−30	−30
D	0	−11
E	−11	+ 9

Potassium cyanide was present in the reaction mixture at a final concentration of 10 mM and all assays were performed at pH 5·5. Assays including EDTA (0·1 mM final concentration) were performed at pH 6·2.

Effects of ethanol. Holden[6] has shown that the linoleate-oxidizing activities of lipoxygenase and haem-proteins are affected differently by ethanol. All fractions were inhibited progressively by ethanol with at least 30% inhibition at 10% (v/v) ethanol. The fractions did, however, show different sensitivities in this test (Table 6).

TABLE 6. EFFECT OF ETHANOL ON LINOLEATE-OXIDIZING ACTIVITY OF FRACTIONS ELUTED FROM CM-CELLULOSE

Fraction	Ethanol (%)		
	10	20	30
	% Inhibition		
B	32	73	95
C	57	100	100
D	55	85	100
E	78	100	100

All fractions were assayed at pH 6·2.

Protein-bound haematin was assayed in the four active peak fractions. The protein was very dilute by this purification stage and the method used was therefore liable to considerable error. Fractions B–E contained 6, 0, 6 and 32 μg/ml protein–bound haematin, each with an error of ±6 μg/ml.

DISCUSSION

Acetone powders of pea seedlings have been shown to contain linoleate oxidizing activity by a polarographic method using ammonium linoleate as substrate. This activity was not obtained when a continuous spectrophotometric method of assay was used, with Tween-solubilized linoleic acid as substrate.[15] The oxidizing activity is very much like that of the enzyme lipoxygenase in its substrate specificity, optimum pH, heat sensitivity, and

[15] BEN-AZIZ, A., GROSSMAN, S., ASCARELLI, I. and BUDOWSKI, P. (1970) *Anal. Biochem.* **34,** 88.

lack of sensitivity to cyanide and EDTA.[1] The linear relationship between extract concentration and oxidizing activity also indicates the presence of lipoxygenase.[1] The possible alternative sources of this activity, the haem proteins, are ruled out on the basis of the above properties and because two model compounds, peroxidase and cytochrome c show very little linoleate oxidizing activity in this assay system over a wide range of concentrations. Thus this work confirms that lipoxygenase is present in plant tissue other than seeds and storage tissue.[6,14] The activity in pea seedlings has been shown to occur in both the leaf and the stem tissue. In contrast to the lipoxygenase activity found in wheat leaves by Holden,[6] the activity in pea seedlings is not acetone-sensitive.

The purification procedure used, based on work by Eriksson and Svensson,[16] and Grossman *et al.*[14] considerably purified the lipoxygenase activity while removing most of the peroxidase activity of the tissue. The partially purified lipoxidase from gel filtration was separated into four active fractions, B–E, by ion-exchange chromatography. Fraction B was eluted by 0·05 M acetate buffer, had pH optima at 5·5, 6·45 and 8·45, contained no detectable haem or peroxidase activity, would not oxidize oleate, and was slightly inhibited by cyanide and slightly stimulated by EDTA. It also behaved like soybean lipoxygenase in the presence of ethanol. These properties suggest that most of the activity in fraction B was due to a lipoxygenase, which is in contrast to the corresponding fraction from alfalfa leaves.[17]

Fraction C, eluted with 0·10 M acetate, had the weakest specific activity and the smallest total activity of the fractions. It behaved like lipoxygenase in its inhibition by ethanol, its optimum pH 6·2, and its substrate specificity but despite containing no haem or peroxidase activity it was inhibited by cyanide and EDTA. The properties of fraction C differ considerably from those of the corresponding fraction from alfalfa leaves and indicate the possibility that it could be a metal containing lipoxygenase.[18]

Fraction D, eluted with 0·15 M acetate, had the greatest specific activity and behaved in all respects like a lipoxygenase (optimum pH 6·2, effect of cyanide, EDTA and EtOH) except that it could utilize oleate as a substrate. The reason for this anomaly is unknown.

Fraction E, eluted with 0·1 M Na_2HPO_4, contained the highest proportion of total linoleate oxidizing activity, and despite its high levels of haem and peroxidase activity, it had properties, e.g. optimum pH 6·2, effect of inhibitors, substrate specificity, which fit well with its containing a lipoxygenase.

The differences in properties between the activities of the four fractions indicate that (at least) four different lipoxygenase iso-enzymes are present in pea seedlings. In a preliminary experiment in which only the early fractions were recovered from gel filtration, subsequent CM-cellulose chromatography revealed activity in fractions B and E only. This may indicate a size difference between the isoenzymes. The relationship of these isoenzymes to those found in pea seeds remains to be investigated.[16,19]

EXPERIMENTAL

Growth and treatment of seedlings. Dwarf pea (*Pisum sativum*, var Meteor) seedlings were grown in moist vermiculite in the dark at 21°. At various times the seedlings were excised above the cotyledons, weighed, cut into pieces and homogenized in 10 vol. of cold acetone (−20°). The extracts were filtered, washed with 10 vol. of cold

[16] ERIKSSON, C. E. and SVENSSON, S. G. (1970) *Biochem. Biophys. Acta* **198**, 449.
[17] BEN-AZIZ, A., GROSSMAN, S., BUDOWSKI, P. and ASCARELLI, I. (1971) *Phytochemistry* **10**, 1823.
[18] TAPPEL, A. L. (1955) *J. Am. Oil Chemists' Soc.* **32**, 252.
[19] HALE, S. A., RICHARDSON, T., ELBE, J. H. VON and HAGEDORN, D. J. (1969) *Lipids* **4**, 209.

acetone, dried and stored in a desiccator at −20°. In certain experiments extracts were made of seedlings that had received continuous cycles of 1 min of red light illumination followed by 29 min darkness. These are referred to as light-grown seedlings.[20]

Chemicals. Linoleic acid, cytochrome-*c* (beef heart Type IV) peroxidase (Horseradish, Type 1) and catalase (bovine liver) were obtained from Sigma Chemical Co. (St. Louis). Lipoxygenase (soybean) was obtained from Seravac Laboratories, Colnbrook, England.

Enzyme assay procedures. Lipoxygenase was assayed by a polarographic method using ammonium linoleate as substrate.[2] The reaction mixture consisted of 0·5 ml 0·5 M acetate buffer (pH 6·2), 0·5 ml ammonium linoleate (6 mM) and H_2O to 2·5 ml. The reaction was started by adding the enzyme and oxygen uptake was followed using an oxygen electrode. A unit U is defined as the uptake of 10 nmol oxygen/min. Peroxidase was assayed by the method of Dicks and Friend.[4] The reaction mixture contained 0·10 ml 0·18 M guaiacol, 0·20 ml 0·03 M H_2O_2 and was made up to a final vol. of 3·0 ml with 0·02 M phosphate buffer (pH 5·6). The reaction was started by adding enzyme and followed as a rise in absorbance at 460 nm. 1 unit (U) is defined as a ΔA of 0·1/min. Catalase activity was assayed as a decrease in absorbance at 240 nm according to Bergmeyer.[21]

Protein determination. The presence of protein in column effluents was monitored by measurement of absorbance at 280 nm. Actual protein concentrations were determined by the method of Lowry *et al.*[22] Protein bound haematin was assayed by the method of Hartree.[23]

Analysis of enzyme reaction products. Reaction mixtures were set up in test tubes as for the assay of lipoxygenase. Lipoxygenase or acetone powder extracts were added to start the reaction and after 25 min at 20°, 1 ml of each mixture was removed and added to 9 ml 80% EtOH. After centrifugation the spectrum of the supernatant was determined. To the remaining 1·5 ml of each mixture, 6 ml $CHCl_3$–MeOH 1:1 was added, followed by 1 ml 0·2 M acetate buffer (pH 4·0). After vigorous shaking, 1 ml was removed from the lower layer, evaporated under N_2, and redissolved in 0·2 ml $CHCl_3$. Samples were applied to a neutral silica gel G TLC plate and run in petrol. (b.p. 60–80)–Et_2O–HOAc (70:30:1). After drying, the lipid product (R_f 0·2) and unchanged linoleic acid (R_f 0·4) were detected with iodine vapour.

Purification. Acetone powder from 10-day-old etiolated pea seedings was solubilized in 30 vols of 0·05 M phosphate buffer pH 7·0. After filtration, the filtrate was treated with $(NH_4)_2 SO_4$. The protein that precipitated between 30 and 50% saturation was redissolved in a small vol. of phosphate buffer and applied to a Sephadex G200 column. Fractions (10 ml) were assayed for protein (A_{280}) and lipoxygenase and peroxidase activity. The fractions with most lipoxygenase activity were pooled and reprecipitated with a 50% saturation $(NH_4)_2SO_4$. The precipitate was redissolved in 10 ml 0·005 M acetate buffer (pH 5·7) and dialysed overnight against this same buffer. The dialysate was then applied to a CM-cellulose column and eluted sequentially with starting buffer (fraction A), 0·05 M acetate (pH 5·7) (fraction B), 0·10 M acetate (pH 5·7) (fraction C), 0·15 M acetate (pH 5·7) (fraction D), and 0·1 M Na_2HPO_4 (fraction E).[14]

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[20] BURDEN, R. S., FIRN, R. D., HIRON, R. W. P., TAYLOR, H. F. and WRIGHT, S. T. C. (1971) *Nature New Biology* **234**, 95.

[21] BERGMEYER, H. U. (1963) *Methods of Enzymatic Analysis* (WILLIAMSON, D. H., trans.), p. 885, Academic, New York and London.

[22] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.

[23] HARTREE, E. F. (1955) *Modern Methods of Plant Analysis* (PEACH, K. and TRACEY, M. V., eds.), Vol. 4, p. 197, Springer, Berlin.