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LIPOXIDASE ACTIVITY OF LEAVES

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Abstract—The peroxidation of linoleic acid by leaf extracts is shown to be catalysed by a lipoxidase-type enzyme.

INTRODUCTION

LIPOXIDASE (lipoxygenase, EC 1.13.1.13) is known to occur in legume seeds, notably soya beans, and in some cereal grains and oil seeds.[1] Its presence in various other plant tissues, including leaves, has also been reported.[2-4] However, Blain *et al.*[5] recently cast doubt on this enzyme being responsible for fatty-acid peroxidizing activity and suggested that haematin compounds are the catalysts. Although haematin compounds are possibly involved in the breakdown of fatty-acid hydroperoxides, i.e. have lipoperoxidase activity,[6] the evidence that they actually catalyse the formation of hydroperoxides by plant extracts is meagre. Much work on fatty-acid peroxidation in plants has been complicated by its being linked to the destruction of carotene[7,8] and chlorophyll.

While studying chlorophyll breakdown in plants, the leaves of many species were examined for their ability to form hydroperoxides from linoleic acid and it was of interest to determine whether the activity found was caused by haematin compounds or by a lipoxidase-type enzyme.

RESULTS

Linoleic Acid Peroxidizing Activity of unfractionated Leaf Extracts

Extracts from the leaves of over forty species from twenty families were tested for their ability to peroxidize linoleic acid.[9] In addition, a study was made of thirty-six members of the Compositae.[10] The activity varied widely from Petty Spurge (*Euphorbia peplus*), in which it was barely detectable, to Wheat cv. Cappelle where the leaves of etiolated seedlings were, on a dry weight basis, about half as active as soya flour.* Barley and rye leaves had less

* Do-soy from British Arkady Co. Ltd., Manchester.

[1] A. L. TAPPEL, in *Lipids and their Oxidation* (edited by H. W. SCHULTZ, E. A. DAY and R. O. SINNHUBER), p. 122, Avi Publishing Company, Westport, Connecticut (1962).
[2] E. V. BUDNITSKAYA, *Biokhimiya* **20**, 614 (1955).
[3] A. M. SIDDIQI and A. L. TAPPEL, *Plant Physiol.* **31**, 320 (1956).
[4] K. S. RHEE and B. M. WATTS, *J. Food Sci.* **31**, 664 (1966).
[5] J. A. BLAIN, J. D. E. PATTERSON and M. PEARCE, *J. Sci. Food Agr.* **19**, 713 (1968).
[6] J. A. BLAIN and E. C. C. STYLES, *Nature* **184**, 1141 (1959).
[7] J. FRIEND and G. J. ACTON, in *Energy Conversion by the Photosynthetic Apparatus*, Brookhaven Symposia in Biology, No. 19, 485 (1966).
[8] J. W. DICKS and J. FRIEND, *Phytochem.* **6**, 1193 (1967).
[9] M. HOLDEN, unpublished.
[10] M. HOLDEN, *Rep. Rothamsted exp. Stn for* 1966, p. 101 (1967).

activity than wheat and in oats it was very small. Leaves of etiolated cereal seedlings were up to three times as active as green leaves of the same age. For convenience, therefore, extracts of etiolated wheat seedlings were used for experiments when an active pigment-free extract was needed.

Mature green leaves that had moderate to high linoleic acid peroxidizing activity included *Urtica dioica* (Urticaceae), *Digitalis purpurea* (Scrophulariaceae), *Solanum dulcamara* (Solanaceae) and several members of the Compositae, including *Arctium lappa*, *Doronicum plantagineum*, *Cirsium arvense* and *Centaurea* sp. Extracts from young leaves of *Phaseolus vulgaris* (Leguminosae) were very active. As the leaves expanded the activity on a wet weight basis steadily decreased, but it remained nearly constant for about 10 days when expressed as amount of activity per leaf.

The activity was retained for several days in leaves kept in polythene bags at 2° and also in frozen leaves, but it was unstable in extracts, particularly when these were well aerated. For example, extracts made by grinding leaves in a mortar were much more active than those made in a homogenizer. An acetate extract (pH 6) of etiolated wheat leaves lost 60 per cent of its activity in 3 hr at 21° and 33 per cent at 2°. Similar losses were found with extracts from green leaves of several other species. Adding ascorbic acid, cysteine, DIECA or Polyclar AT during grinding did not stabilize the activity.

Peroxidation of Peroxide-Free and Peroxide-Containing Linoleic Acid

With leaf extracts hydroperoxide was formed at similar rates from slightly peroxidized and peroxide-free linoleic acid. With haematin compounds (haemoglobin;* cytochrome *c*;† haemin) under the same assay conditions, hydroperoxide was formed from linoleic acid already containing some peroxide but none from peroxide-free acid. Table 1 compares an extract from etiolated wheat leaves with haemoglobin.

TABLE 1. PEROXIDATION OF LINOLEIC ACID BY AN EXTRACT OF ETIOLATED WHEAT LEAVES AND BY HAEMOGLOBIN

Time of incubation (min)	EEL reading			
	Peroxide-containing linoleic acid		Peroxide-free linoleic acid	
	Hb§	Leaf extract§	Hb	Leaf extract
5	1·50	1·20	0	1·10
10	2·72	2·37	0	2·22
15	4·05	3·80	0	3·75

§ Hb—200 μg (8μg/ml reaction mixture); leaf extract—0·05 ml.

Hydroperoxide was formed in proportion to the amount of wheat-leaf extract used for the tests. This was also found with extracts of green wheat seedlings and with the extracts of many other leaves. However, with a few species, particularly some with low activity, increasing the amount of extract did not proportionately increase the rate of hydroperoxide formation. This may be because the extracts contain inhibitors of peroxidation such as have been found in

* Koch Light Laboratories Ltd., from sheep, twice cryst.
† Boehringer Corporation (London), Ltd., from horse heart, cryst.

lucerne[11] and in tomato fruit.[5] Boiled extracts of green and etiolated wheat leaves and green leaves of red and white clover strongly inhibited peroxidation catalysed by haematin compounds though not by soya lipoxidase.

Tappel[12] showed that for haematin compounds the rate of oxidation of linoleate was proportional to the square root of the catalyst concentration. Using slightly peroxidized linoleic acid this was confirmed for haemoglobin, with amounts up to 8 μg/ml (0·36 μg haematin/ml) in the reaction mixture. With 40 μg/ml there was a lag period of several min before hydroperoxide was formed and with 80 μg/ml none at all was formed during 20 min incubation. This effect is similar to that found by Banks *et al.*[13] with cytochrome *c*.

When leaf extracts were boiled for 2 min the peroxidizing activity was completely inactivated when tested on both peroxidized and peroxide-free linoleic acid. In contrast, boiled haemoglobin, cytochrome and haemin solutions catalysed hydroperoxide formation in peroxide-containing linoleic acid at almost the same rate as unboiled (Table 2). However, haematin compounds boiled in the presence of leaf extracts had smaller activity. This did not seem to be due to actual inactivation but was caused by the presence of inhibitory substances mentioned above.

TABLE 2. EFFECT OF BOILING ON FATTY-ACID PEROXIDIZING ACTIVITY OF LEAF EXTRACTS AND HAEMATIN COMPOUNDS

Catalyst	EEL reading after 10 min incubation	
	Not boiled	Boiled (2 min)
Extract of etiolated wheat leaves (0·05 ml)	6·65	0
Extract of green *Urtica* leaves (0·1 ml)	2·50	0
Haemoglobin (200 μg)	3·10	3·00
Cytochrome *c* (75 μg)	2·10	1·75

Effect of Ethanol on Peroxidation

Peroxidation by lipoxidase is more sensitive than peroxidation by haematin compounds to ethanol in the reaction mixture. The standard reaction mixture contained 2 per cent of ethanol but increasing the concentration even slightly diminished the amount of hydroperoxide formed by soya lipoxidase and by leaf extracts. Peroxidation by haemoglobin or haemin was unaffected by concentrations of ethanol up to 22 per cent. Figure 1 compares the effect of various concentrations of ethanol on the formation of hydroperoxide by soya lipoxidase*, an extract of etiolated wheat leaves and by haemoglobin.

Effect of Other Inhibitors of Peroxidation

Lipoxidase activity is not inhibited by comparatively high concentrations of potassium cyanide;[8] the peroxidizing activity of wheat extracts was not inhibited by concentrations of up to 40 mM KCN. Several other known inhibitors of linoleic acid peroxidation were tested, but

* Seravac Laboratories (Pty.) Ltd., lyophilized powder.

[11] A. BEN AZIZ, S. GROSSMAN, P. BUDOWSKI, I. ASCARELLI and A. BONDI, *J. Sci. Food Agr.* **19**, 605 (1968).
[12] A. L. TAPPEL, in *Autoxidation and Antioxidants* (edited by W. O. LUNDBERG), Vol. 1, p. 325, Interscience, John Wiley, New York (1961).
[13] A. BANKS, E. EDDIE and J. G. M. SMITH, *Nature* **190**, 908 (1961).

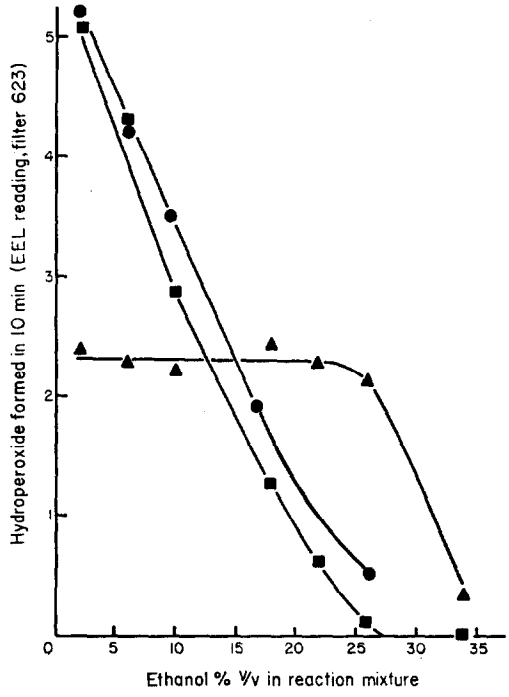

FIG. 1. EFFECT OF ETHANOL ON THE PEROXIDATION OF LINOLEIC ACID AT pH 6 BY AN EXTRACT OF ETIOLATED WHEAT LEAVES, SOYA LIPOXIDASE AND HAEMOGLOBIN.

■ Wheat-leaf extract, 0·05 ml. ▲ Haemoglobin, 200 μg. ● Soya lipoxidase, 750 μg.

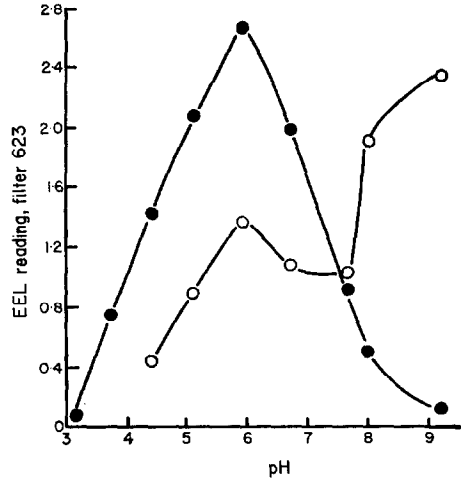

FIG. 2. EFFECT OF pH ON THE PEROXIDATION OF LINOLEIC ACID BY AN EXTRACT OF GREEN WHEAT LEAVES AND BY HAEMOGLOBIN.

● Wheat-leaf extract, 0·3 ml, 10 min incubation; ○ 200 μg haemoglobin, 5 min incubation. Buffers: pH 3·1–6·7—acetate; pH 7·7 and 8·0—phosphate; pH 9·2—borate.

the experiments did not provide any useful information for distinguishing between lipoxidase and haem catalysis in leaf extracts. EDTA could not be used because it interfered with the determination of hydroperoxide in the ferric thiocyanate method.

Effect of pH

Figure 2 compares the effect of pH on peroxidation by an extract of green wheat leaves and by haemoglobin. The leaf extract showed a well-defined optimum near pH 6 and the activity declined sharply on either side. The haemoglobin also showed a peak of activity near pH 6 but in addition catalysed rapid peroxidation at pH 8 and above. The reaction mixtures with pH values below 8 were slightly turbid because the linoleic acid was present as an emulsion, but at pH 8 and above the mixtures were clear and this coincided with increased peroxidation by the haemoglobin.

The pH optimum for peroxidation by leaf extracts varied a little between species but always lay between pH 6 and 7 and was lower than for soya lipoxidase (above pH 8) determined under the same conditions.

Haematin Compounds in Leaves

More haematin was required for peroxidation of linoleic acid under the test conditions than the amount that is probably present in most plant extracts. Hill and Scarisbrick[14] gave a figure of 32 μg/g fresh wt. for wheat leaves and this was one of the larger values quoted by Davenport.[15] The amount of wheat-leaf extract needed for tests in the present work would, on this basis, contain 0·4 μg if all was extracted from the tissues. The reaction mixture (25 ml) would therefore at most contain 0·016 μg haematin/ml; peroxidation with such an extract was, however, slightly faster than with 0·4 μg haemin/ml. Using the method of Paul *et al.*[16] haematin compounds could not be determined in extracts of etiolated wheat leaves because the differences between large blanks and the test samples were so small.

Much of the haematin of plant tissues is in the form of peroxidase and catalase. The haematin in these enzymes contributes only about 1 per cent to their weight so that quite large amounts (up to 40 μg/ml reaction mixture) are needed to be comparable with haemoglobin and cytochrome *c*. Catalase* and peroxidase† were used in a few experiments but neither of them catalysed the peroxidation of linoleic acid to the extent expected from the haematin content; further work is needed to clarify their possible role as fatty-acid peroxidizing factors.

Concentration of the Fatty-Acid Peroxidizing Factor

Much of the activity was associated with the plastid fraction of etiolated wheat leaves. It could be extracted by sodium dodecyl sulphate (SDS) or Triton X-100 and the amount in the extracts was greater than was apparently present in the original sample (Table 3). Two treatments with either detergent extracted about the same amount of activity but the first treatment with Triton always extracted more than that with SDS. The residue after two extractions with Triton still had far greater activity than appeared to be in the starting material. One treatment with SDS extracted about half the activity from the residue after Triton extraction.

* Sigma Chemical Co., from bovine liver, twice cryst.
† Preparation from horse-radish root with PZ 770.

[14] R. Hill and R. Scarisbrick, *New Phytologist* **50**, 98 (1951).
[15] H. E. Davenport, in *Biochemists' Handbook*, p. 1033 (1961).
[16] K. G. Paul, H. Theorell and Å. Åkeson, *Acta Chem. Scand.* **7**, 1284 (1953).

TABLE 3. EXTRACTION OF FATTY-ACID PEROXIDIZING FACTOR FROM PLASTID FRACTION OF ETIOLATED WHEAT LEAVES

	SDS		Triton X-100	
	Enzyme units	% original activity	Enzyme units	% original activity
Plastid preparation	42·4	100	42·4	100
First extract	46·5	110	60·5	143
Second extract	21·6	51	10·4	24
Residue	15·7	38	67·5	160
Total recovered	83·8	199	138·4	327

8 ml plastid preparation from etiolated leaves of Cappelle wheat seedlings shaken for 5 min with either 8 ml 0·5 per cent Triton X-100 or 8 ml 0·5 per cent sodium dodecyl sulphate (SDS). Pellet after centrifuging shaken with 8 ml 0·5 per cent solution of detergent. Residue suspended in 0·2 M acetate buffer, pH 5·9.

The active factor in detergent extracts could be concentrated by precipitating with ammonium sulphate; acetone precipitation caused almost complete inactivation. It could also be concentrated by ultrafiltration with little loss of activity.

CONCLUSION

Linoleic acid peroxidizing activity is widely distributed in leaves and the active factor is heat-labile and alcohol-sensitive. Leaf extracts peroxidize peroxide-free linoleic acid whereas haematin compounds do not. Leaf extracts show a marked pH optimum between 6 and 7 and are almost inactive at pH 8 and above where haematin compounds actively catalyse peroxidation. The possibility is not ruled out that in some leaves fatty-acid peroxidation is catalysed by haematin compounds, but all the evidence suggests that the active factor in the leaves of many species is a lipoxidase-type enzyme.

EXPERIMENTAL

Plant Material

Samples of cereal seeds were kindly given by Dunns Farm Seeds, Salisbury. Seedlings were grown on moist filter paper in glass tanks and used when 8–10 days old. Etiolated seedlings were harvested in the light so contained a trace of chlorophyll. Leaves of other plants were from various sources both cultivated and wild; mature leaves were mostly used.

Preparation of Extracts

Leaves were ground in a mortar with NaOAc buffer, 0·2 M, pH 5·9, 4 ml/g wet wt., and a small amount of fine sand. The extract was squeezed through cloth and cell-wall debris and sand removed by centrifuging briefly at low speed.

The plastid fraction from etiolated wheat leaves was made by centrifuging the crude extract at 6000 rpm for 10 min (about 3000 *g*). The pellet was washed once with acetate buffer, 0·2 M, pH 5·9, and then suspended in a small volume of the buffer and stored at −18°.

Determination of Linoleic Acid Peroxidizing Activity

The formation of linoleate hydroperoxide was followed by the ferric thiocyanate method. The reaction mixture which was incubated at about 21° totalled 25 ml and contained 5 mg linoleic acid in 0·5 ml ethanol, NaOAc buffer, pH 6·0, 0·04 M, and catalyst. Samples (1 ml) were removed at zero time and then at 5 min intervals for 15 min and added to 10-ml high grade absolute ethanol containing 0·1 ml HCl. After adding the reagents the absorption was read in an EEL colorimeter with filter 623. The blank reading given by a control without catalyst kept for the same time was deducted.

Slightly peroxidized linoleic acid was usually used but for some experiments acid from freshly opened ampoules was used within a few hours and this contained no detectable hydroperoxide.

One arbitrary unit of enzyme activity is the amount that gives a reading on the EEL colorimeter, with filter 623, of 1·0 after 10 min incubation under the conditions described above.