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ALDRIN EPOXIDASE FROM PEA ROOTS

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Abstract—The membrane system which epoxidizes aldrin to dieldrin in pea roots was separated from polyphenolic inhibitors and soluble peroxidase by rapid anaerobic gel filtration. Epoxidase activity was strongly inhibited by cytochrome-c, cyanide, electron acceptors and polyphenols and was enhanced by chelating and solubilizing agents which inhibit cytochrome P450 systems.

INTRODUCTION

Pesticides are metabolized by a wide variety of plants *in vivo* [1]. Although recent study has established that pesticide metabolism in animals occurs in the complex of microsomal mixed-function oxidases, little is known of the systems which metabolize pesticides in plants. Cell-free extracts of plants *N*-demethylate urea herbicides [2] and oxidize organochlorine insecticides, converting aldrin to dieldrin by epoxidation [3–5]. These reactions require NADPH and oxygen and thus appear to be similar to animal mixed-function oxidase reactions. Electron transport activities including cytochromes P450 [6,7] and b_3 [8,9] and NADH and NADPH-cytochrome-c oxidoreductases and diaphorases have been measured in plant microsomes [8, 10]. Peroxidase, which is able to perform at least some of the biotransformations of pesticides occurring in plants, has also been found in plant microsomal material [10,11].

We shall present evidence to show that the system effecting epoxidation of aldrin to dieldrin in pea roots is distinct from other oxidative systems and appears to consist of an NADPH-cytochrome-c oxidoreductase and a peroxidase-like terminal oxidase.

RESULTS AND DISCUSSION

Rapid gel filtration provided material free from low MW inhibitors and soluble peroxidase (Fig. 1). Anaerobic preparation under N_2 reduced yellowing and the formation of oxidized and reduced components, as peroxidases and polyphenol oxidases were separated from their substrates. The method was suitable for other root homogenates including those from broad bean which normally darken very rapidly.

Epoxidase

The technique was designed to achieve the maximum speed of separation and provide a large quantity of material, rather than to resolve protein. Protein fractions and centrifuged pellets were white or pale yellow when prepared anaerobically, in contrast to the usual deep yellow or brown colour of pea root protein prepared aerobically.

Turbidity was a useful indicator of the particulate material containing epoxidase activity as shown in Fig. 1. The epoxidase activity was totally excluded on Sephadex G-100 (Fig. 1). About 70% of the total epoxidase activity obtained from the column could be sedimented at 105000 *g* in 2 hr. Epoxidation was inhibited 20% by NADH at 200 μM but no inhibition could be detected at 1 mM. Activity was stimulated 40, 50, 80 and 100% by NADPH at 50, 100, 500 and 1000 μM respectively. Low levels of activity were consistently obtained without the addition of the reduced nucleotides. It has been

Table 1. Substances which affect aldrin epoxidase activity

Substance	Concn (mM)	% Of control
Cytochrome-c	0·1%	24
Menadione	1·0	22
Benzoquinone	1·0	43
Iodonitrotetrazolium violet	0·1	23
Tetrazolium salt	0·5	48
Neotetrazolium chloride	0·5	67
Methyl viologen	1·0	88
KCN	1·0	19
Hydroquinone	1·0	35
Pyrogallol	1·0	31
Orcinol	1·0	63
Aniline	1·0	60
p-Bromophenol	1·0	42
p-Bromoanisole	1·0	52
Phenol	1·0	102
Cinnamic acid	1·0	135
Phenobarbital	1·0	130
Deoxycholate	1·0	122
Triton X100	0·05%	110
Phenanthroline	1·0	154
α,α^1 Dipyridyl	1·0	135

Activity was the amount of dieldrin produced from 10 μg of aldrin in 2 hr incubation with 1 ml of gel filtered pea root extract and an NADPH generating system.

suggested that a similar effect observed in beans was due to endogenous NADPH [4]. However this explanation is unsatisfactory for gel-filtered preparations. We suggest that other components of the system may be reduced before or during preparation as indicated later.

A number of substances capable of withdrawing electrons from the system were inhibitors (Table 1), including cytochrome-c, menadione, benzoquinone and the tetrazolium salts. Iodonitro tetrazolium chloride was the most easily reduced and the strongest inhibitor. As the redox potential became more negative the inhibition decreased and thus methyl viologen was only a weak inhibitor. The epoxidase was very sensitive to cyanide. Previous reports [3,4] did not show this clearly, as soluble peroxidase which has a strong affinity for cyanide, had not been removed. The inhibition by cyanide suggested that the pea root epoxidase was different from other plant mixed-function oxidases which were unaffected or only slightly affected by cyanide [2,12]. The epoxidase was also inhibited by hydroquinone, pyrogallol, orcinol, aniline, *p*-bromophenol and *p*-bromoanisole. Phenol itself was not an inhibitor. Cinnamic acid and phenobarbital stimulated epoxidation. We previously showed [13] that pretreatment of peas with aniline and phenobarbital altered their rate of epoxidation *in vivo* and *in vitro*, and phenol pretreated plants were unaffected. This may not be due to the type of enzyme induction which occurs in animal liver microsomes as we have suggested [13], but the result of direct stimulation by phenobarbital and inhibition by aniline of the epoxidase. The effect of these compounds in tissue extracts closely resembled that obtained by pretreatment of intact plants, thus demonstrating that studies on tissue extracts may be of value in determining the mechanism of pesticide metabolism in intact plants. The results also suggest that oxidative systems in plant roots may be very sensitive to chemical changes in the environment of the root including the presence of pesticides.

Chelating and solubilizing agents which damage cytochrome P450 systems increased epoxidation activity in pea root extracts (Table 1). The stimulatory effects of deoxycholate, triton X-100, phenanthroline and α,α'dipyridyl upon epoxidation were almost the exact reverse of their effects upon oxidative demethylation of urea herbicides in cotton (2). The cotton *N*-demethylase required NADPH and was inhibited by cytochrome-c and menadione but was stimulated by cyanide. Thus the plant epoxidase and *N*-demethylase appear to have a similar oxidoreductase component but have terminal oxidases with antagonistic properties.

NADPH-cytochrome-c oxidoreductase

Stimulation of epoxidation by NADPH and inhibition by cytochrome-c and menadione suggest that NADPH-cytochrome-c oxidoreductase may be a functional component of the pea root epoxidase, as it is in animal microsomes. Inhibition of epoxidation by other electron acceptors indicates the importance of electron transport in the system. Cytochrome P450 could not be detected in the pea microsomes in the present study.

NADPH-cytochrome-c oxidoreductase activity was widespread throughout pea root homogenates. Much of the activity was soluble and not associated with the epoxidizing membranes (Fig. 1). The soluble reductase activity appearing in the 30000 *g* supernatant (Table 2) was unstable

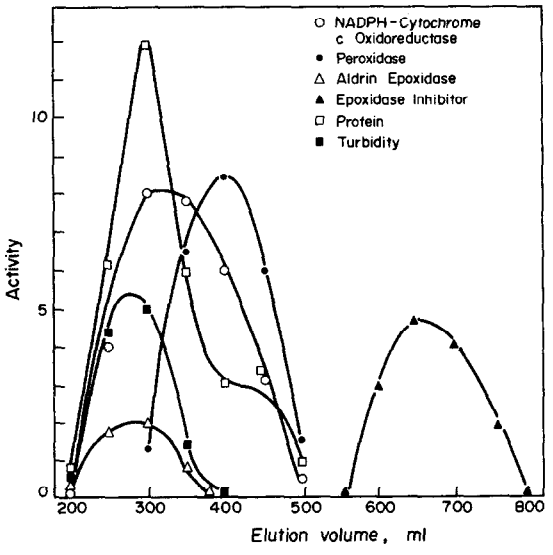

Fig. 1. Partial purification of aldrin epoxidase by rapid gel filtration. Activities: NADPH–cytochrome-c oxidoreductase (A550/min/ml × 10^2), peroxidase (A423 (reduced–oxidized) × 10^2), aldrin Epoxidase (μg dieldrin/2 hr/ml × 10), epoxidase inhibitor (% inhibition × 10^{-1}/0·1 ml), protein (mg/ml × 10), turbidity (A700 × 10).

and showed considerable loss in 24 hr. The activity sedimenting below 30000 *g* apparently occurred in large membrane fragments, was more stable and related well to epoxidation. Preparations made with sucrose showed a slight increase in activity of both reductase and epoxidase in the membranes sedimenting below 30000 *g*. It is possible that the reductase was being solubilized from membranes during preparation and that sucrose may have partly protected against solubilization by stabilizing some membrane vesicles. The solubilized form of the reductase may be less stable because it is the most degraded form.

Table 2. Distribution of NADPH-cytochrome-c oxidoreductase and aldrin epoxidase in gel filtered homogenates

Homogenate fraction	NADPH-Cytochrome-c oxidoreductase Control (8 hr)	Sucrose (6 hr)	Sucrose (30 hr)
2000 *g* pellet	0·001	0·008	0·006
5000 *g* pellet	0·003	0·010	0·008
15000 *g* pellet	0·004	0·012	0·009
30000 *g* pellet	0·003	0·007	0·004
30000 *g* supernatant	0·040	0·024	0·014

Homogenate fraction	Aldrin epoxidase Control (8–10 hr)	Sucrose (6–8 hr)
2000 *g* pellet	0·015	0·030
5000 *g* pellet	0·020	0·040
15000 *g* pellet	0·025	0·035
30000 *g* pellet	0·030	0·035
30000 *g* supernatant	0·14	0·12

Control preparation was made using 0·1 M KPi buffer pH 7. A second preparation was made with the same buffer containing 0·3 M sucrose. Eluent from the gel column (200–300 ml; Fig. 1) was collected and centrifuged as indicated for 20 min. Fractions were analysed for oxidoreductase (A_{550}/min/ml) and epoxidase (μg dieldrin/2 hr/ml) at the time stated, after homogenization.

Peroxidase

The inhibition of epoxidation by cyanide and polyphenols suggests that peroxidase may be a component of the system. However peroxidase was found almost entirely in a soluble form in pea roots (Fig. 1). Rungie and Wiskitch [8] measured a turnip microsomal peroxidase and distinguished it from cytochrome b_3 by its spectral shifts with cyanide.

Attempts to concentrate the peroxidase, resulted in aggregation, and Sephadex G-100 chromatography showed that both the peroxidase and NADPH-cytochrome-c oxidoreductase found in the soluble fraction were heterogeneous in size. These observations indicated that the peroxidase in fresh pea root preparations may be a solubilized membrane component. Also a fresh preparation of the pea root peroxidase may be adsorbed on to an interface made by spreading ether on the solution of peroxidase in buffer. On evaporating the Et_2O the peroxidase was freed and returned to the solution with only small losses, suggesting a lipophilic property. This could not be repeated with commercial horseradish peroxidase or aged preparations of the same pea root peroxidase. It has been reported that the endoplasmic reticulum of beetroot parenchyma rapidly disintegrates on sectioning [14] and that solubilization of microsomal components occurs [15]. Thus it is possible that only a very small proportion of the oxidative ability of the plant is retained in tissue homogenates.

Table 3 sets out the absorption maxima of pea root peroxidase in difference spectra with dithionite compared with horseradish peroxidase and peroxidases from clover and pumpkin root extracts. Fresh pea root peroxidase has a sharp absorption peak at 423 nm and two very broad

Table 3. Absorption maxima of soluble peroxidases from pea, clover and pumpkin roots compared with commercial horseradish peroxidase

Source	Difference spectra α(nm)	β(nm)	γ(nm)
Reduced–Oxidized			
Pea			
Fresh	572	537	423
Aged	560		435
Fresh clover			423
Fresh pumpkin			428
Horseradish	560		438
Reduced + Cyanide–Oxidized			
Pea			
Fresh	565	535	428
Aged			433
Fresh clover			432
Fresh pumpkin			430
Horseradish	564	535	433

A solution of 0·01% horseradish peroxidase or 30000 *g* supernatants of plant root homogenates were reduced with solid sodium dithionite and then made to 1 mM with KCN.

bands at 537 and 572 nm. Addition of cyanide broadens the 423 nm peak and shifts it to 428 nm. Cyanide also sharpens the spectrum from 500 to 600 nm and peaks appear at 535 and 565 nm. In contrast, horseradish peroxidase has a broad peak at 438 nm with dithionite which shifts in the opposite direction to 433 nm with cyanide. Cyanide sharpens the 500–600 nm region of horseradish peroxidase and two peaks appear at 535 and 564 nm replacing the broad peak at 560 nm of the reduced form.

Attempts to purify pea root peroxidase by concentration using ammonium sulphate or a pressure membrane, followed by further Sephadex chromatography resulted in a change to a spectral form which more closely resembled horseradish peroxidase. In preparations containing both forms, only the 423 nm form would adsorb on to an Et_2O–H_2O interface. This form therefore appears to be distinct from previously reported forms of peroxidase.

Fresh clover root preparations contained the 423 nm form and a small amount of a second form as a shoulder at about 445 nm. In fresh pumpkin root preparations the peroxidase was at the limit of detection appearing to be in a form similar to horseradish peroxidase. We previously showed that intact pea and clover roots converted aldrin to dieldrin while pumpkin roots were much less active [13]. Table 4 shows that this was also true in tissue extracts and that peroxidase, measured both spectrally and by peroxidation of phenol, follows a similar pattern. NADPH-cytochrome-c oxidoreductase also shows some variation with species. It has been suggested [16] that the wide variation of pesticide metabolizing ability among different animals may partly depend upon the amounts of some of the components of electron transport in the microsomes. It appears that there may also be a general relationship between pesticide metabolism and the amounts of haemoproteins and flavoproteins in different plant species.

Table 4. Oxidative activities in root tissue from several plants

	Pea	Clover	Pumpkin
Aldrin epoxidase	0·040	0·035	0·003
NADPH–cytochrome-c oxidoreductase	0·10	0·08	0·05
Peroxidase	0·095	0·085	0·015
Phenol peroxidase	4·6	4·4	0·8

Crude, filtered root homogenates containing 1 g root tissue/1·5 ml 0·1 M KPi buffer, pH 7 and 5% Polyclar were used for analysis. Activities were: aldrin epoxidase (μg dieldrin/2 hr/ml), NADPH–cytochrome-c oxidoreductase (Δ550/min/ml), peroxidase [(Δ423 or 428 (reduced–oxidized)] and phenol peroxidase (Δ400/min/ml).

Epoxidase inhibitor

Normally at least a five-fold increase in epoxidation activity appeared after gel filtration. The increase appeared to be due to the removal of heat stable low MW inhibitors (Fig. 1). A soluble inhibitor of epoxidation has been reported in broad beans [5]. Plant root extracts are known to contain a wide variety of polyphenols and quinones and we have shown that such substances are potent inhibitors of epoxidation (Table 1).

Polyphenols from pea root extracts were prepared by concentrating a boiled, filtered, crude homogenate *in vacuo* and chromatographed on silica gel G using *n*-BuOH–HOAc–H_2O (6:1:2). Five broad bands appeared on spraying with Folin and Ciocalteu's Reagent. If the extract was first passed through a Polyclar column only two bands appeared on TLC but these were strong inhibitors of epoxidation. They also were able

to reduce cytochrome-c, having a redox potential of +0·34 V with respect to the saturated calomel electrode. These reducing substances caused difficulty in the measurement of NADPH–cytochrome-c oxidoreductase activity in crude homogenates.

All protein fractions from a crude extract prepared in the presence of air contained a substance which showed a broad fluorescence peak at 440 nm when excited with light at 250 nm. Fresh preparations which were rapidly gel-filtered under N_2 did not form polyphenols capable of reducing cytochrome-c but still contained polyphenolic inhibitors of epoxidation. In this case protein fractions had a fluorescence maximum of 325 nm when excited at 250 nm. We are uncertain about the nature of this fluorescent component which closely follows NADPH-cytochrome-c oxidoreductase activity throughout the preparation but it appears to have a redox state which changes according to the method of preparation, in a similar manner to the low MW polyphenols.

The formation of reduced polyphenols during preparation could occur by reduction of quinones by a flavoprotein reductase. This also suggests a mechanism for their inhibition of epoxidation by withdrawing electrons. A microsomal NADPH-cytochrome-c oxidoreductase will transfer electrons from NADPH to menadione and indirectly to other quinones [17]. Alternatively reducing power in the crude preparation could be generated by a peroxidation reaction [18].

CONCLUSION

Rapid anaerobic gel filtration is a useful method for preparing plant root extracts. Polyphenols and quinones which inhibit aldrin epoxidation are removed and there is no formation of the reduced polyphenols which makes the measurement of NADPH-cytochrome-c oxidoreductase activity difficult. Electron transport artifacts, due to reduction of components such as flavoprotein and haemoprotein during preparation, are less likely to occur. The partially purified epoxidase closely resembles the system which attacks aldrin in the intact pea root, clearly showing that the epoxidase itself is not an artifact of preparation. Properties of the epoxidase which have now been determined suggest that it consists of a membrane bound oxidoreductase and a terminal oxidase which resembles peroxidase. Fig. 2 shows a possible scheme for aldrin epoxidation.

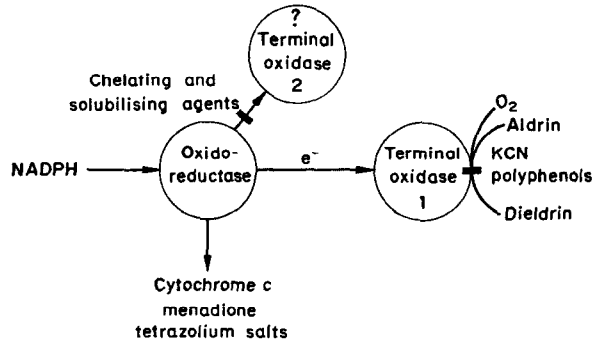

Fig. 2. Electron transport scheme for aldrin epoxidation. An oxidoreductase transfers electrons from NADPH to the terminal oxidase which combines aldrin with oxygen to form dieldrin. Electron acceptors compete with epoxidation by withdrawing electrons from the system. The terminal oxidase resembles peroxidase in being inhibited by cyanide and polyphenols. A second system such as a terminal oxidase with properties like cytochrome *P*450 may compete with the epoxidase. Removal of this by chelating or solubilizing agents thus increases electron flow to the epoxidase.

Peroxidase measured spectrally was found entirely in the soluble fraction of pea root extracts. However plant peroxidases are well known to exist in a variety of forms. It appears that at least one form is bound to the membranes and takes part in aldrin epoxidation. The significance of the large amount of soluble peroxidase and soluble NADPH-cytochrome-c oxidoreductase is uncertain but these may be membrane components solubilized during preparation.

EXPERIMENTAL

Plant materials. Peas, *Pisum Sativum* cv. Progress No. 9, clover, *Trifolium subterraneum* cv. Clare, and pumpkin *Cucurbita pepo* cv. Yates Queensland Blue were grown as previously reported [13].

Tissue extracts. Roots of 2-week-old plants were washed in H_2O, partially dried and homogenized in degassed 0·1 M KPi buffer pH 7 (100 g/150 ml) with 5% Polyclar under N_2. In one series of expts the buffer contained 0·3 M sucrose as indicated. The mixture was filtered through a Buchner funnel containing a circle of Miracloth under N_2. Crude filtrate (100 ml) was transferred to a large Sephadex G-100 column equilibrated in the correct degassed buffer under N_2.

The entire procedure following homogenization could be accomplished in about 10 min without contamination by O_2 which if accidentally introduced, produced immediate yellowing.

The column (7 cm dia) gave a flow rate of *ca* 5 ml/min with a void vol. of 200 ml. Fractions (10 ml) were collected and every 5th tube analysed for epoxidase and other activities. The first 5–6 tubes containing substantial epoxidase activity were pooled and used to examine properties of the epoxidase or to locate inhibitors. Membrane fractions were prepared by centrifuging for 20 min and pellets were resuspended in centrifuge tubes with a loose fitting perspex pestle in 10% of the original vol. of buffer. Activities were calculated as units/ml of pooled extract.

Epoxidase and inhibitor assays. To a 12 mm dia glass bottle was added 0·35 μmol NADPH, 3·5 μmol glucose-6-phosphate, 0·2 units of glucose-6-phosphate dehydrogenase and any test substance or fraction containing inhibitor (0·1 ml) in a total vol. of 0·5 ml of KPi buffer. To test the effects of nucleotides the NADP concn was varied with the same generating system and NADH was added in the absence of a generating system. Aldrin (10 μg) was added to each bottle in methyl cellosolve (10 μl) and reaction was initiated by adding 1 ml of root extract. Reaction mixtures were shaken at 25° for 2 hr and the reaction was terminated by freezing in Me_2CO–ice. Samples were stored at −10° until analysis. Pesticides were extracted by shaking the reaction mixture with redist *iso*PrOH (1 ml) and hexane (1 ml). The upper phase (5 μl) was injected into a gas chromatograph equipped with a ^{63}Ni EC detector. Conditions for GLC were as previously reported [13]. Standards of aldrin and dieldrin distributed in H_2O:*iso*-PrOH:hexane (3:2:2) were used to determine the amount of dieldrin produced in 2 hr of incubation.

NADPH–Cytochrome-c oxidoreductase assay. NADPH (20 μM), cytochrome-c (20 μM), Antimycin A (0·8 μg), KCN (1 mM) and extract (0·1–1 ml) were mixed in a total vol. of 1·5 ml buffer. NADPH was added rapidly after KCN to initiate the reaction measured as the increase in *A*550. (Adapted from Joshi *et al.* [19]).

Peroxidase by difference spectra. A baseline was established by scanning identical samples in 2 cuvettes. Sufficient solid sodium dithionite was added to reduce the peroxidase and the *A* of the reduced form at 423 nm was recorded in pea and clover root preparations, at 428 nm in pumpkin root extracts and 438 nm in solns of commercial horseradish peroxidase. All forms were identified as peroxidase by the shift of the Soret peak and sharpening of the spectrum in the 500–600 nm range with formation of peaks at *ca* 535 and 565 nm upon adjusting the soln of the reduced form to 1 mM KCN.

Phenol peroxidase. To 0·5–2 ml of extract was added 1 ml of 1 mM phenol in KPi buffer in a final vol. of 3 ml. H_2O_2 (0·1 ml, 3%) was added and the initial increase in *A*400 was measured. No activity occurred in the absence of H_2O_2 or in the presence of 1 mM KCN.

Turbidity. The *A*700 was measured against KPi buffer. *Protein* was measured by the method of Lowry [20].

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