Effect of the Safener Dichlormid on Maize Peroxygenase and Lipoxygenase*

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S-Ethyl-N,N-dipropylthiocarbamate (EPTC) was oxidized into its corresponding sulfoxide by microsomal fractions from etiolated maize seedlings. This reaction is catalyzed by a hydroperoxide-dependent enzyme, identified as a peroxygenase. The hydroperoxides formed from fatty acids by a lipoxygenase are efficient co-substrates of the EPTC sulfoxidation. The effects of the safener dichlormid on the peroxygenase and lipoxygenase activities were studied *in vitro* and *in vivo*. *In vitro*, the safener is not an inhibitor of these enzymes. Dichlormid seems to act, *in vivo*, by modulating the amounts of peroxygenase and lipoxygenase in treated plants.

Introduction

According to Casida *et al.* [8] the sulfoxide of the thiocarbamate EPTC is a more potent herbicide than the parent compound. So far, however, no enzymatic systems have been described achieving the sulfoxidation of EPTC in plants. We present here evidence that the peroxygenase, an original hydroperoxide-dependent oxidase, is a good candidate to fulfil such a role.

Corn is protected from injury due to EPTC by the safener dichlormid. The mode of action of this safener involves an enhancement of the mechanisms of detoxification of EPTC (*e.g.*, increased GSH levels and GSH-transferase activities) [9, 10]. It was also suggested that dichlormid could increase the rate of EPTC sulfoxidation [11]. In order to test this latter hypothesis, we have studied, the influence of dichlormid on the sulfoxidase activity of the peroxygenase. Since the plant peroxygenase is strictly hydroperoxide-dependent for its activity, we have also investigated the effect of the safener on lipoxygenase, the enzyme catalyzing the formation of fatty acid hydroperoxides in plants.

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Materials and Methods

Maize seeds (Dekalb XL-25-A) were grown and treated with EPTC and dichlormid as described in [1]. Microsomal fractions were prepared from 4 days old etiolated seedlings according to [2].

Determination of enzyme activities

EPTC (1 mM) was incubated, for 1 h at 30 °C, with the microsomal fraction (0.7 mg protein), in 0.1 M sodium citrate buffer, pH 5.5 (final volume: 0.1 ml), in the presence of 10^5 dpm [^{14}C]EPTC (10 mCi/mmol). The reaction was then stopped by freezing in liquid nitrogen. After saturation of the reaction mixture with solid NaCl, unlabelled EPTC and EPTC sulfoxide were added as carriers and extraction was performed by 3 × 1 volume of diethyl ether. The combined organic phases were dried and evaporated with a stream of argon. The residue was applied on TLC plates (60 F_{254}, Merck) which were developed in hexane/acetone (1:1, v/v). EPTC and EPTC sulfoxide were visualized by UV and the corresponding spots scrapped off the plate and counted.

Methiocarb oxidation was performed as already described [3]. Oxidation of thiobenzamide by the maize peroxygenase was measured according to the method described by Cashman and Hanzlik [4] for the mammalian flavin monooxygenase. Oleic acid epoxidation was assayed according to [5]. The lipoxygenase activity was measured spectrophotometrically at 234 nm [6]. Protein concentrations were determined [7] using serum albumin as standard.

Results and Discussion

Characteristics of plant peroxygenases

In mammalian systems, sulfide substrates are sulfoxidized by microsomal cytochrome P450-dependent monooxygenases [12] and by flavin-containing monooxygenases [13]. Both classes of enzymes are membrane-bound and require NADPH and molecular oxygen as cosubstrates. In plants, mixed-function monooxygenases have also been described and their involvement in the metabolism of some pesticides has been demonstrated [14–18]. Peroxidases, which are very active in plants, are also known to detoxify several xenobiotics [19]. Apart from all these oxidative enzymes, a quite unusual oxidase named: peroxygenase is also present in plants. This enzyme was first described by Ishimaru and Yamazaki [20a, b], in microsomes of germinating pea seeds, as a hydroxylase catalyzing the hydroxylation of aromatic heteroatom-containing substrates (such as indoles, phenols, primary arylamines). Recently, we have shown that this peroxygenase exhibits also epoxidase and sulfoxidase activity (Fig. 1).

The peroxygenase is a membrane-bound enzyme [2] which does not require any cofactors such as NAD(P)H [3] for its activity. It is a hemoprotein which possesses spectral properties distinct from those of cytochrome P450 or peroxidases [5, 21]. Its name peroxygenase derives from the fact that the source of the oxygen incorporated into the reaction products originates exclusively from hydroperoxides, and not from molecular oxygen, regardless of the reaction catalyzed, *i.e.*, hydroxylations [20], sulfoxidations [22] or epoxidations [5].

Occurrence of EPTC sulfoxidase in maize microsomes

As a sulfoxidase, the peroxygenase catalyzes the oxidation of a wide variety or sulfur-containing compounds (Fig. 2) and EPTC should be a good substrate. Indeed, preliminary experiments indicated that EPTC was a competitive inhibitor in the peroxygenase catalyzed epoxidation of oleic acid (Table I). Doerge [23] showed the oxidation of arylsulfides by the microsomal fractions of germinating pea seeds. We have investigated the sulfoxidation of EPTC by maize seedlings.

Maize seedlings were found to convert [^{14}C]labelled EPTC into its sulfoxide (Fig. 3a, b and c); no further oxidation into the sulfone could

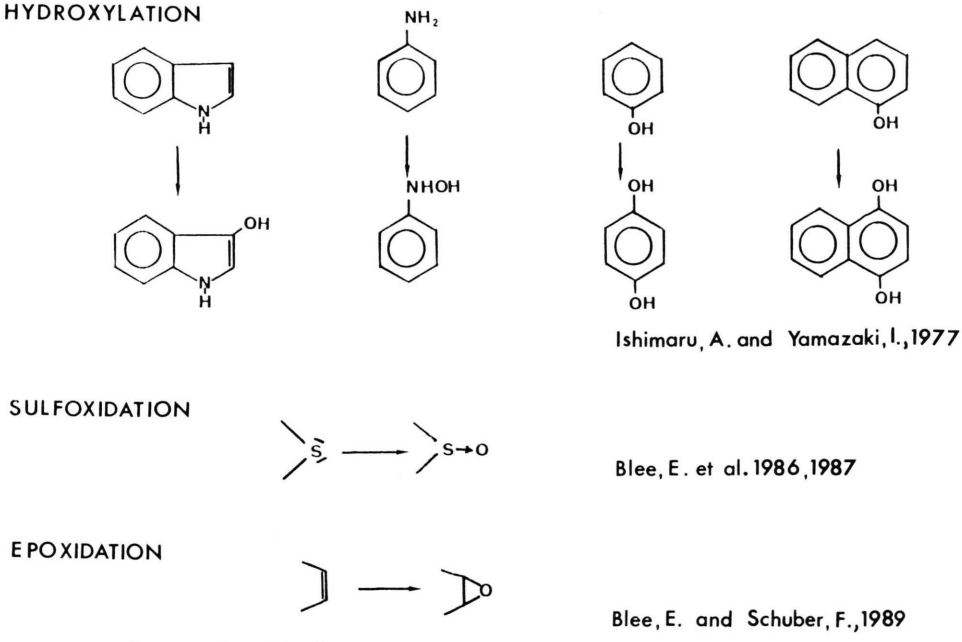

Fig. 1. Reactions catalyzed by the peroxygenase.

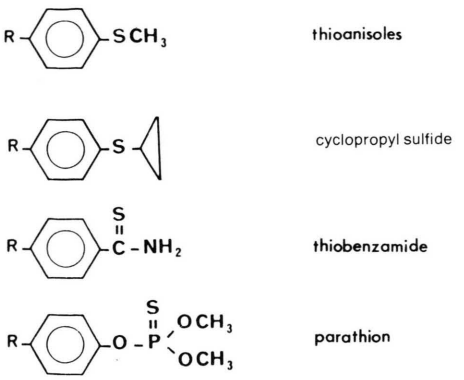

Fig. 2. Sulfur-containing compounds oxidized by the peroxygenase.

Table I. Inhibition of oleic acid epoxidation by EPTC.

Addition	Inhibition (%)
Control	0
EPTC 100 μM	54
EPTC 200 μM	80

Soybean peroxygenase was incubated in the presence of 20 μM [^{14}C]oleic acid, 100 μM cumene hydroperoxide and concentrations ad indicated of EPTC. Oleic acid epoxide formation was estimated as described in [5].

be observed. The highest activity was found in the shoots and, in this organ, it was associated with the microsomal fraction. The oxidative ability of the membranes was destroyed when microsomes were heated to 60 °C for 2 min. Sulfoxidation of EPTC did not require cofactors such as NAD(P)H, which seems to rule out the participation of classical P450- or FAD-dependent monooxygenases.

Characteristics of the sulfoxidase activity

We have further investigated the exact nature of the enzyme involved in such sulfoxidation by using methiocarb (4-(methylthio)-3,5-xylyl methyl carbamate) as a model substrate, since EPTC is too volatile and unstable for such a study.

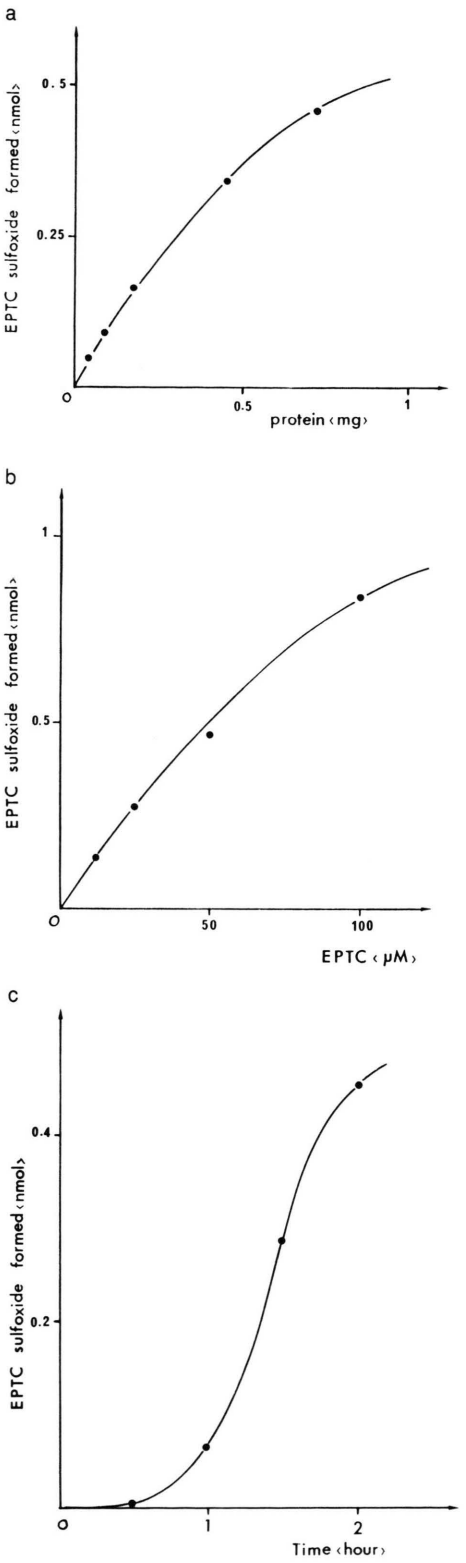

Fig. 3. Formation of EPTC sulfoxide catalyzed by maize microsomes as a function of protein (a) and EPTC (b) concentrations and time (c). Maize microsomes were incubated in 0.1 M citrate buffer (pH 5.5) in the presence of [^{14}C]EPTC.

Microsomes prepared from various plants (maize, carrot, potato and particularly peas and soybean) were found to efficiently catalyze oxidation of methiocarb into its sulfoxide (Fig. 4). This sulfoxidation reaction followed classical Michaelis-Menten kinetics. Methiocarb sulfoxidation was inhibited by mercaptoethanol, sodium azide and sodium bisulfite and was insensitive to cyanide ions and to EDTA [3]. A similar pattern was found for the sulfoxidation of aldicarb by soluble corn roots extracts and the enzyme involved in the metabolism of this insecticide exhibits certain characteristics of plant peroxidases [24]. However, the sulfoxidase did not act as a peroxidase because: i) H_2O_2 was not a co-substrate, ii) no effect of inhibitors of peroxidative reactions (KCN, catalase) could be observed and iii) classical peroxidase such as horseradish peroxidase did not metabolize methiocarb [2]. In agreement with the study on oxidation of EPTC, the formation of methiocarb sulfoxide did not require any cofactors such as NAD(P)H, but was totally inactive if hydroperoxides (cumene or fatty acid hydroperoxides) were omitted in the medium.

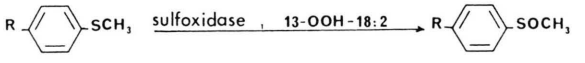

Fig. 4. Methiocarb sulfoxidation by the peroxygenase.

All together, these results seem to rule out the participation of any cytochrome P450 and FAD-dependent monooxygenases or peroxidases in the sulfoxidation of EPTC but strongly suggest the involvement of peroxygenase in such a reaction.

Effect of dichlormid on peroxygenase activity

Sulfoxidation followed by the cleavage by the glutathione S-transferase system were postulated to be the detoxication processes of EPTC [8, 9, 25–27]. Dichlormid acts in corn to prevent injury from EPTC. Several hypotheses were made on the mode of action [28] of this safener, mostly involving the stimulation of the detoxication processes. Another suggestion was an increased rate of EPTC sulfoxide formation [11]. If this latter hypothesis is correct, one could expect an enhancement of the peroxygenase activity by the antidote. Thus, we studied the biotransformation of EPTC in dichlormid treated or untreated maize.

Data from Table II show that the S-oxygenation of EPTC in microsomes prepared from treated maize was reduced compared to that of untreated plants. A very similar effect of the safener on maize peroxygenase was observed if this activity (present both in treated or untreated plants) was measured spectrophotometrically in the presence of cumene hydroperoxide and thiobenzamide as substrates (Table II). However, dichlormid did not act directly on the peroxygenase since under *in vitro* conditions, dichlormid at $10^{-7} \times 10^{-5}$ M did not alter the enzyme activity (the activity was determined radiochemically as [^{14}C]EPTC sulfoxide formation or spectrophotometrically using thiobenzamide as substrate). Thus, in strong contrast with the former hypothesis, dichlormid does not stimulate the formation of EPTC sulfoxide. The inhibition observed seems to be due to a lesser quantity of the peroxygenase present in the treated maize rather than to a direct action of the antidote on the enzyme.

Table II. Peroxygenase and lipoxygenase activity in treated and untreated maize.

Treatment[a]	Peroxygenase activity[b] [%]	Peroxygenase activity[c] [%]	Lipoxygenase activity[d] [%]
Control	100	100	100
EPTC	62	67	44
Dichlormid	46	47	66
EPTC + Dichlormid	43	46	63

[a] Maize seedlings were treated with 10^{-5} M dichlormid and/or 10^{-4} M EPTC for 4 days.
[b] Peroxygenase was measured as EPTC sulfoxidation activity.
[c] Peroxygenase was measured as thiobenzamide activity.
[d] Lipoxygenase was measured as described in the text.

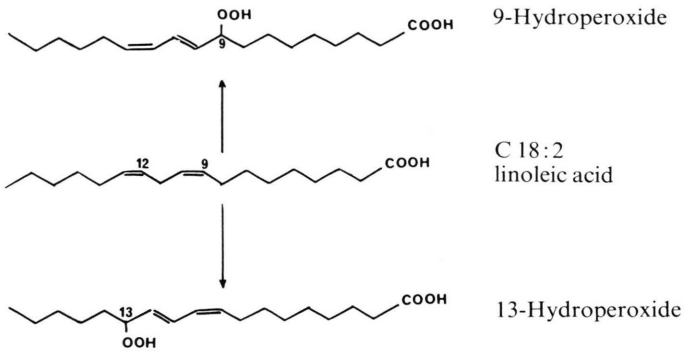

Fig. 5. Reactions catalyzed by the lipoxygenases.

Effect of dichlormid on the lipoxygenase activity

Peroxygenase activity is strictly hydroperoxide-dependent [22]. *In vivo,* hydroperoxides are generated non-enzymatically, *e.g.* under conditions of stress (induced for example by fungal pathogen agressions [29 and references therein], by herbicides [30 and references therein]) or enzymatically by lipoxygenases. A modification of the lipoxygenase activities may result in a regulation of the peroxygenase. For this reason, we have investigated the possible effect of dichlormid on the lipoxygenase activity.

Lipoxygenase (EC 1.13.11.12) is an ubiquitous enzyme in plants that utilizes molecular oxygen in the conversion of polyunsaturated fatty acids, containing a *cis, cis*-1,4 pentadiene system, to conjugated hydroperoxydiene derivatives (Fig. 5). We found in our maize seedlings a lipoxygenase mostly soluble and active at acid pH (5.5–6). Lipoxygenase converts polyenoic fatty acids to a number of isomeric products. The maize lipoxygenase converts linoleic acid mainly to 13-hydroperoxy 9,11-octadecadienoic acid (67%) whereas smaller amounts of 9-hydroperoxy 10,12-octadecadienoic acid are formed. By means of chiral-phase HPLC [31], we have shown that about 98% of the main product was in the *S*-configuration, whereas only 2% was *R*. The same strong stereospecificity was observed for the side-product 9-hydroperoxy 10,12-octadecadienoate (89% *S*-configuration and 11% in the *R*-configuration) (see Fig. 6).

Dichlormid treated maize exhibited a lower lipoxygenase activity than the untreated plants (Table II). No direct effect of the safener on lipoxygenase could be detected under *in vitro* conditions.

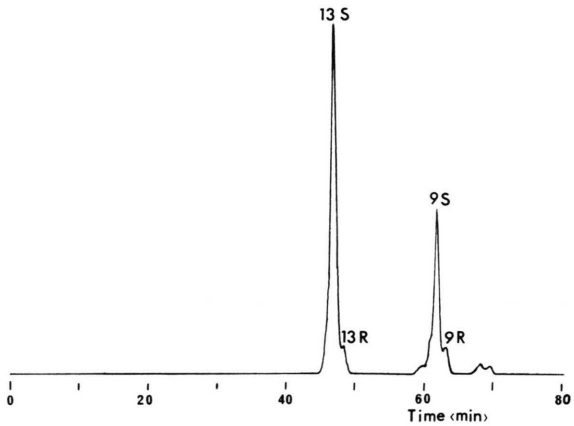

Fig. 6. Elution profile, on chiral HPLC, of the stereo- and regio-isomers of the hydroxyoctadecadienoates obtained by reduction of the hydroperoxides formed from linoleic acid by the maize seedlings lipoxygenase.

Only an artificial inhibition could be observed when lipoxygenase was measured spectrophotometrically; but this was due to the strong absorbance of dichlormid at 234 nm resulting in a reduced sensitivity in the detection of the produced hydroperoxide. Similar inhibition was noticed by [32] for several compounds.

Conclusion

EPTC is oxidized into its sulfoxide by maize microsomes. This oxidation is catalyzed by a peroxygenase strictly hydroperoxide-dependent for its activity and requiring lipoxygenase activity for the production of its cosubstrates. We have investigated the action of the safener dichlormid on these en-

zymes and have found a decrease of their apparent activities in treated plants. Dichlormid is not an inhibitor of these enzymes *in vitro,* and most likely seems to act, *in vivo,* by modulating the amounts of peroxygenase and lipoxygenase in treated maize.

It is obvious that a lesser amount of the herbicidal EPTC sulfoxide will be formed in maize treated by dichlormid. One can speculate that protective effect of dichlormid could be due to this reduced formation of toxic herbicide metabolite followed by its increased metabolism in treated maize.

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