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Characterization of epoxide hydrolase activity in *Alternaria alternata* f. sp. *lycopersici*. Possible involvement in toxin production

Epoxide hydrolase in Alternaria alternata f. sp. lycopersici

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Abstract

Using *trans*-diphenylpropane oxide (tDPPO) as a substrate, we measured epoxide hydrolase (EH) activity in subcellular fractions of *Alternaria alternata* f. sp. *lycopersici* (Aal), a fungus that produces host-specific toxins. The activity was mainly (>99.5%) located in the soluble fraction (100,000 × g supernatant) with the optimum pH at 7.4. An increase of toxin production between days 3 and 9 found in a Aal liquid culture over a 15 days period was concomitant with a period of high EH activity. EH activity remained constant during the same period in an *Alternaria alternata* culture, a fungus which does not produce toxin. *In vivo* treatment of Aal culture with the peroxisome proliferator clofibrate stimulated EH activity by 83% and enhanced toxin production 6.3 fold. Both 4-fluorochalcone oxide (4-FCO) and (2S,3S)-(-)-3-(4-nitrophenyl)-glycidol (SS-NPG) inhibited EH activity in vitro with a I_{50} of 23 ± 1 μM and 72 ± 19 μM, respectively. The possible physiological substrate 9,10-epoxystearic acid was hydrolyzed more efficiently by Aal sEH than the model substrates *trans*- and *cis*-stilbene oxide (TSO and CSO) and *trans*- and *cis*-diphenylpropane oxide (tDPPO and cDPPO).

Key words: Epoxide hydrolase, fungus, inhibitor, AAL toxins

Abbreviations: CSO, *cis*-stilbene oxide; cDPPO, *cis*-diphenylpropene oxide; EH, epoxide hydrolase; 4-FCO, 4-fluorochalcone oxide; oxide; SS-NPG, (2S, 3S)-(-)-3-(4-nitrophenyl)-glycidol; tDPPO, *trans*-diphenylpropene oxide; TSO, *trans*-stilbene oxide.

Introduction

Epoxide hydrolases (EHs) (EC 3.3.2.3) convert epoxides to diols by addition of water. The cloning of EHs from different origins enabled authors [1, 2] to perform sequence homology analysis and classify them in the group of enzymes known as the α/β hydrolase fold family [3]. The broad spectrum of the mammalian soluble EH (sEH), in addition to the fact that hepatic microsomal EH (mEH) is induced by a variety of foreign compounds, suggests their involvement in xenobiotic metabolism [4, 5]. The tissue specific regulation of sEH in mice might indicate its different physiological roles [6], including participation in the metabolism of endogenous compounds, since epoxides of fatty acids are hydrolyzed more rapidly than many other substrates [7].

Croteau and Kolattukudy [8] were the first authors to report the presence of EH in a plant, which was located in a 3,000 × g particulate fraction from

homogenates of apple skin. A sEH has also been purified from soybean [9] and both enzymes have been proposed to participate in synthesis of cutin monomers. Indeed, they are able to produce diols of fatty acids which are incorporated in plant envelopes [10]. More recently, inducible EHs have been cloned from mouse eared cress (*Arabidopsis thaliana*) and potato (*Solanum tuberosum*) [11, 12].

EHs have also been detected in fungi. Kolattukudy and Brown [13] partially purified an EH from *Fusarium solani pisi* and fungal EHs have been used in organic synthesis for enantioselective hydrolysis [14–17]. However, little information is available on these enzymes and their physiological significance remains to be established. They might be important in plant-fungus interaction. Diols of fatty acids induce a cutinase of *F. solani pisi*, which facilitates the penetration of the fungus in the plant [18]. *Alternaria alternata* f sp. *lycopersici* (Aal), is a fungal pathogen which causes the stem canker in tomato [19], a disease elicited by the toxins produced by the fungus (AAL toxins) [20]. *Alternaria alternata* (Aa) is a fungus morphologically similar to Aal, but which does not produce detectable toxin. The presence of two pairs of vicinal diols free or esterified in the AAL toxins (Figure 1) suggests the possible involvement of EH in their synthesis.

The presence of EH activity in *Alternaria alternata* f sp. *lycopersici*, its substrate specificity and the effect of inhibitors on the activity *in vitro* are described in this paper. In an attempt to understand the role of EH in this fungus, the correlation between toxin production and EH activity in both Aal and Aa liquid cultures and the effect of *in vivo* treatment of an EH inducer (clofibrate) on both Aal EH activity and toxin production were evaluated.

Materials and methods

Chemicals. KCN, $Na_2B_4O_7.10H_2O$, KH_2PO_4 and ethyl 2-(4-chlorophenoxy)-2-methylpropionate (clofibrate) were purchased from Aldrich Chemical Company (Milwaukee, WI). 3-(4-Nitrophenyl)glycidol (SS-NPG and RR-NPG) and BSA were purchased from Sigma (St. Louis, MO). Naphthalene-2,3-dicarboxaldehyde (NDA) was obtained from Molecular Probes, Inc (Eugene, OR). 4-Fluorochalcone oxide (4-FCO) was synthesized as reported previously [21]. [2-^{3}H]-*trans*-1,3-Diphenyl propene oxide and [2-^{3}H]-*cis*-1,3-Diphenyl propene oxide ([^{3}H]-tDPPO and [3H]-cDPPO) were synthesized according to Borhan *et al.* [22]. [2-^{3}H]-*trans*-Stilbene oxide and [2-^{3}R]-*cis*-stilbene oxide ([^{3}H]-TSO and [^{3}H]-CSO) were synthesized according to Mullin and Hammock [23]. [1-^{14}C]-9,10-Epoxystearic acid was synthesized from [^{14}-C]-oleic acid (DuPont-New England Nuclear, Boston, MA, USA) using *m*-chloroperoxybenzoic acid. [1-^{14}C]- Methyl-9,10-epoxystearate was synthesized by methylation of [1-^{14}C]-9,10-epoxystearic with diazomethane. All solvents used were HPLC grade (Fisher Scientific).

Subcellular fractionation. Fungal mycelium (2–5 grams) was homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 0.1 M phosphate buffer (pH 7.4) containing 250 mM sucrose, 1 mM EDTA, 15 mM β-mercaptoethanol and 1 mM phenylmethylsulfonylfluoride. The homogenate was filtered through cheesecloth and centrifuged for 25 min at 10,000 $\times$ g at 4°C. The pellet was resuspended in 0.1 M phosphate buffer (pH 7.4), aliquoted and stored at -80°C. A part of the supernatant was aliquoted and stored at -80°C, the rest was centrifuged at 100,000 $\times$ g for 65 min. The supernatant (soluble fraction) was aliquoted and stored at -80°C, the pellet (microsomes) was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 30% glycerol and stored at -80°C. The protein concentration of the samples was determined with the Biorad protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

Determination of EH activity. EH activity was measured with 10,000 g supernatant (5 μg of protein) using radiolabeled tDPPO as already described [22], after incubation for 10 min at 27°C. For the subcellular location of the activity, the amount of protein used per incubation was 5 μg for crude extract and 10,000 g supernatant and 68, 8 and 660 μg for 10,000 g pellet, soluble fraction and microsomes, respectively. Optimum pH was determined after incubation of protein with 100 μM of tDPPO in phosphate buffer (pH 4.5–7.4) and tris buffer (pH 7.8–9.3), with a 0.1 M ionic strength. For the substrate specificity study, EH activity was measured using radiolabeled substrates according to Pinot et al. [24] with 9,10-epoxystearic and methyl-9,10-epoxystearate, Mullin and Hammock [23] with TSO and CSO and Borhan et al. [22] with tDPPO and cDPPO. All the assays were run in 0.1 M phosphate buffer (pH 7.4) at 27°C for 10 min with a substrate concentration of 100 μM. The amount of protein from the soluble fraction in

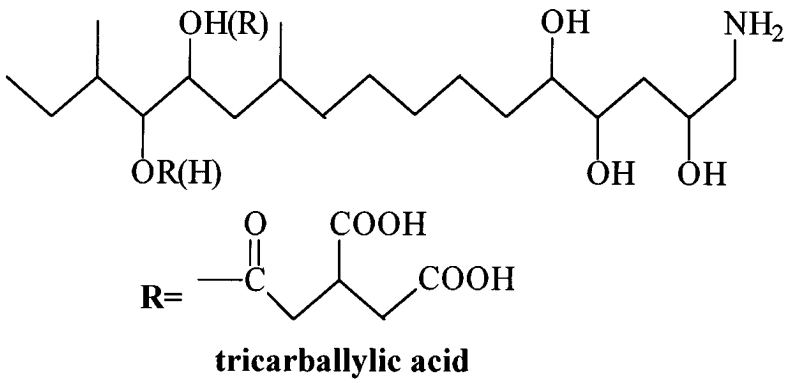


Figure 1. Structure of TA AAL toxins.

each incubation was: 5 μg (TSO, CSO, tDPPO), 12 μg (cDPPO), 16 μg (9,10-epoxystearic acid and the corresponding methyl ester). All assays were shown to be linear for both protein concentration and time. For inhibition studies, the 10,000 g supernatant was pre-incubated with concentration ranging from 1 to 50 μM for 4-FCO and from 10 to 150 μM for SS-NPG and RR-NPG. Controls were pre-incubated with solvent (ethanol) only. After 10 minutes, the substrate (tDPPO) was added and the reaction was allowed to proceed.

Fungal cultures. Alternaria alternata f sp. *lycopersici* and *Alternaria alternata* were grown in a pectin liquid medium [25] at 25°C. The fungi were harvested at 2–15 days after inoculation, according to each experiment. The mycelial suspension was filtered in cheesecloth and squeezed to eliminate the excess water. The mycelium was immediately prepared for measurement of EH activity or kept at −80°C for further analysis. EH activity was measured with 5 μg of protein from the 10,000 × g supernatant incubated with 100 μM tDPPO for 10 min at 27°C. An aliquot of the liquid medium (2 ml) was filtered in 0.45 *m*m nylon filter and analyzed for toxin production (TA AAL toxin) by the HPLC/NDA method. For induction studies, clofibrate (in ethanol at 1 mM final concentration) was added to cultures which were 2 or 3 days old and the cultures harvested at day 6. Ethanol was added to the controls.

Measurement of toxin production. KCN (300 μmol) was added to a 3 ml vial contained 10 μl of sample (liquid medium), followed by 260 μL of 0.05 M $Na_2B_4O_7$. $10H_20$ (pH 8.5) and 150 μmol of NDA. The sample was mixed thoroughly and the reaction mixture incubated at 60°C for 15 minutes in a ReactiTherm heating module (Pierce Chemical). The analysis was performed using a HPLC Beckman System Gold system with Ultracarb 5 ODS 30 (4.5 × 10 mm, Phenomenex) column, coupled to a Perkin Elmer 650 Fluorescence Detector (Ex 420 nm, 10 nm slit, Em 490 nm). The mobile phase consisted of 56% acetonitrile (44% 50 mM H_3PO_4 adjusted to pH 2.8 with NH_4OH). TA AAL toxin, purified in our laboratory according to Caldas et al. [25] and shown to be at least 95% pure based on the ^{1}H-NMR spectrum, was used as standard.

Results and discussion.

EH activity in extracts from Aal grown in liquid culture was measured in different subcellular fractions with tDPPO (Table 1). The highest specific activity was found in the 10,000 × g supernatant. When reported as total activity, the results show that after the 100,000 × g centrifugation, the activity is mainly (>99.5%) located in the soluble fraction. This corroborates previous work performed with another fungus, *Fusarium solani pisi* [13]. Mammalian EH activity is also mostly located in the soluble fraction. In a recent work, Borhan et al. [22] measured the metabolism of five different substrates by four soluble and three microsomal EHs from different organisms. They found that the specific activity of the microsomal EHs were at least two orders of magnitude lower than the specific activity of the soluble enzymes for the substrates examined. Except for the substrate specificity study which was performed with the soluble fraction (100,000 × g), all activity measurement in this work were done with the 10,000 × g supernatant and

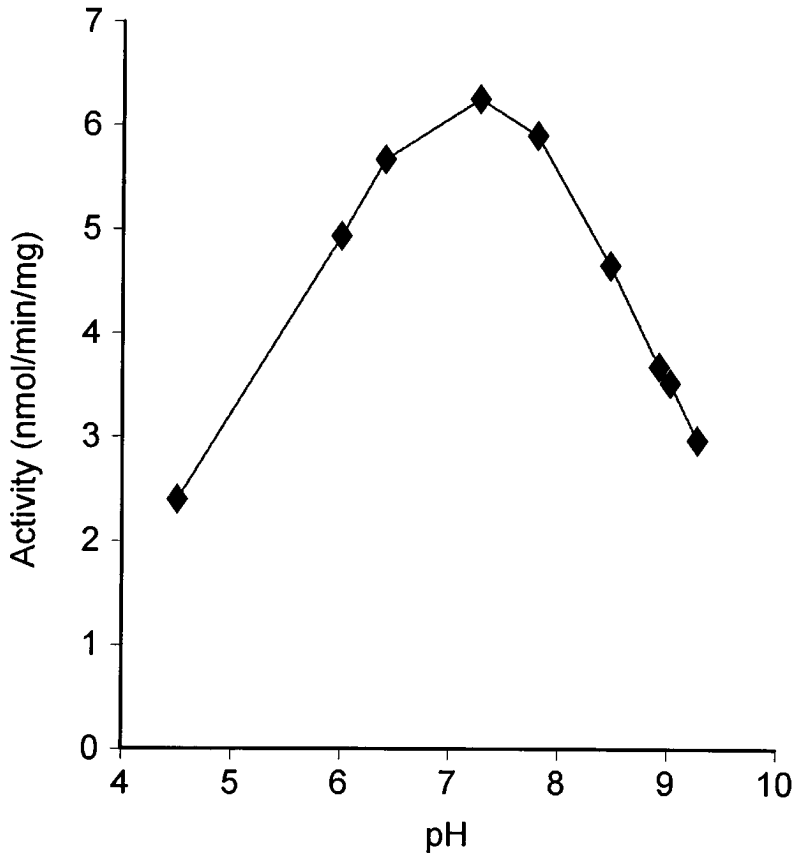


Figure 2. Effect of pH on sEH activity from *Alternaria alternata* f sp. *lycopersici.*

Table 1. Epoxide hydrolase activity in subcellular fractions from *Alternaria alternata* f sp. *lycopersici*

Fraction	Total activity		Specific activity
	nmol/min	%	(nmol/min/mg)
Crude extracts	490	100	3.92
10,000 × g supernatant	479	98	5.71
10,000 × g pellet	8	1.7	0.24
100,000 × g supernatant (soluble fraction)	478	99.8	3.99
100,000 × g pellet (microsomes)	0.8	0.2	0.03

the enzymatic system designated as soluble epoxide hydrolase (sEH).

In order to determine optimum pH, specific activity of sEH was measured at pH ranging from 4.5 to 9.3 (Figure 2). Hydrolysis of tDPPO was maximum at pH 7.4. Interestingly, this pH differs from the optimum pH from *Fusarium solani pisi* (pH 9) [13] and is similar to the optimum pH reported for plant and mammalian sEH (approximately 7.4) [5, 9].

During the infection of tomato by Aal, the fungus produces host specific toxins (AAL toxins) which are aliphatic chains with two pairs of vicinal diols free or esterified [25, 26] (Figure 1). EH activity and toxin production were measured over a 15 days period in a liquid culture of *Alternaria alternata* f sp. *lycopersici* (Aal) and *Alternaria alternata* (Aa), a non toxin producer. As shown in Figure 3, in Aal, there is a peak of sEH activity at days 3 and 6, period in which toxin production increases drastically. At day 9, when sEH activity has already dropped, toxin production stabilized. In Aa, EH activity remained constant during the same period. The fact that there is a change in sEH activity during the log phase of toxin production in Aal culture while EH activity remains practically constant for the non toxin producer (*A. alternata*), suggests a possible relationship between epoxide hydrolase activity and toxin biosynthesis. sEH may be important

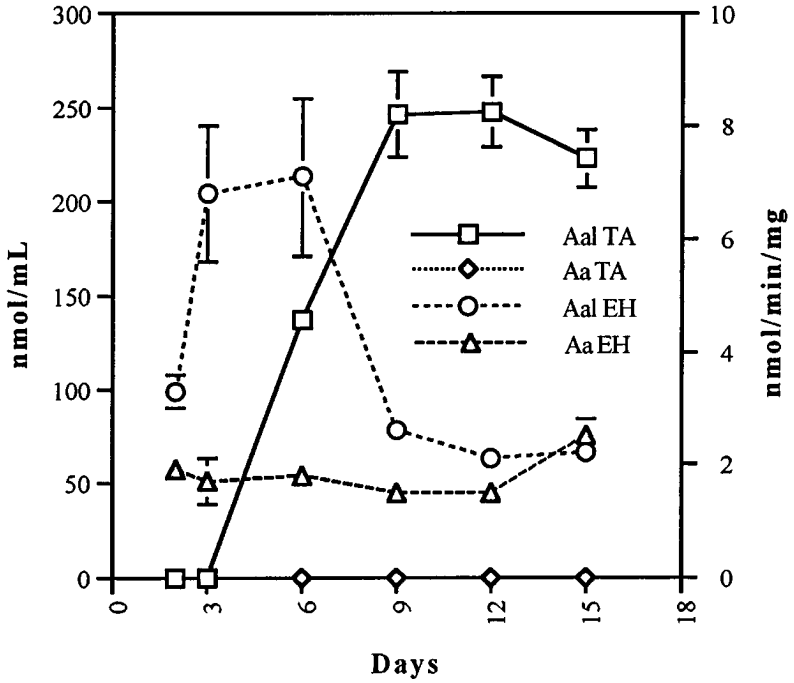


Figure 3. sEH activity and toxin production in liquid culture of *Alternaria alternata* f sp. *Lycopersici* (Aal) and*Alternaria alternata* (Aa).

in the early stage of toxin biosynthesis, as enzyme activity increases before toxin production could be detected.

A better understanding of the physiological role of sEH in Aal may be gained by studying the effect of inducers and inhibitors. Aal was grown in media containing 1 mM of clofibrate, a peroxisome proliferator shown to induce mammalian sEH [27]. Clofibrate treatment stimulated sEH activity by 83% (3.27 ± 0.5 nmol/min/mg protein in controls and 6 ± 1.4 in treated samples). This concomitant induction of sEH activity and stimulation of toxin production is in favor of the sEH participation in biosynthesis of toxins. However, the effect of this treatment was more pronounced on the toxin production which was enhanced 6.3 fold (9.4 ± 1.0 nmol/ml in controls and 59.0 ± 9.1 nmol/ml in treated samples). The mechanism of action of this peroxisome proliferator is still unclear, however it has been proposed that it occurs via the activation of a group of transcription factors, the peroxisome proliferators activated receptors (PPAR) [28]. It is known that peroxisome proliferators cause a wide range of physiological effects [27]. Enhancement of toxin production certainly could result from many events possibly unrelated to the induction of sEH.

SS-NPG and 4-FCO have been shown to inhibit sEHs, but not mEH, from different organisms [11, 12, 21, 29]. We studied the effect of these compounds by measuring hydrolysis of tDPPO after pre-incubation of the 10,000 $\times$ g supernatant of Aal extracts with different concentrations of RR-NPG, SS-NPG and 4-FCO. The latter two compounds inhibited sEH activity and inhibition increased with concentration (Figure 4). I_{50} of 23 ± 1 μM and 72 ± 19 μM for 4-FCO and SS-NPG, respectively, were determined from these experiments. No alteration of activity was observed with the *1R,3R* isomer of NPG, even at a concentration of 300 μM (data not shown). Studies performed with sEHs from mammals and plants [11, 12, 29] have shown that RR-NPG is a much less potent inhibitor than the SS enantiomer. The mechanism of the inhibition of EH by these compounds is still unknown, but according to the catalytic mechanism of EHs [30, 31] it is assumed that these inhibitors act as tight binding substrates.

Substrate selectivity in Aal sEH was investigated by measuring the hydrolysis of different substrates by the soluble fraction (100,000 $\times$ g). As shown in Table 2, *trans*-stilbene oxide (TSO), a model substrate for studies of mammalian sEH, was hydrolyzed twice as fast as its *cis* isomer (CSO). tDPPO and cDPPO, which

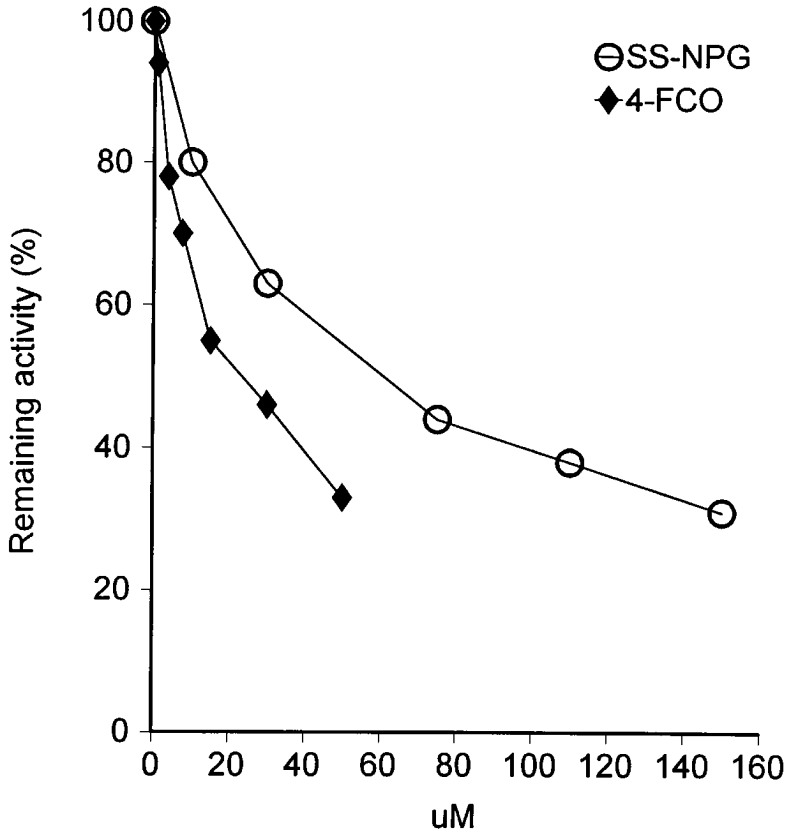


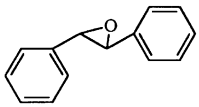
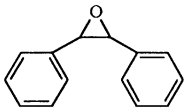
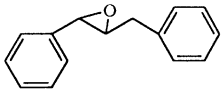
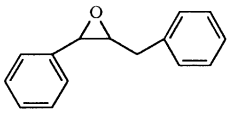
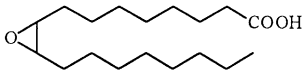
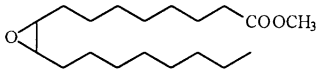
Figure 4. Inhibition of sEH activity from *Alternaria alternata* f sp. *lycopersici* by SS-NPG and by 4-FCO. Results are expressed as percent of activity measured after pre-incubation with solvent only: 6.9 ± 0.6 nmol/min/mg protein (SS-NPG) or 6.5 ± 0.8 nmol/min/mg protein (4-FCO). Data are mean of three independent measurements performed in triplicate.

have been recently developed as surrogate substrates in the laboratory [22], differ from TSO and CSO by the presence of an additional methylene group. The presence of this methylene resulted in an increase of specific activity by one order of magnitude and by a loss of selectivity for the *trans* and *cis* isomer. The highest activity was measured with the physiological substrate 9,10-epoxystearic acid. The methyl ester of 9,10-epoxystearic acid was hydrolyzed at a 10-fold lower rate than the free acid. This observation indicates that a free carboxyl group might be important for the positioning of the substrate in the active site.

In contrast, however, Borhan et al. [22] showed that all mammalian sEHs tested hydrolyzed tDPPO between 3.8 and 15.2 times faster than 9,10-epoxystearic acid. It has been shown that 9,10,18-trihydroxystearic acid, which results from the hydrolysis of 18-hydroxy-9,10-epoxystearic acid, induces cutinase, an enzyme that facilitates the penetration of the fungus in the plant [18]. Diols of fatty acids have antimicrobial activities [32, 33, 34]. The fact that the fungal sEH described here hydrolyses preferentially the physiological substrate 9,10-epoxystearic acid suggests that this enzyme might have a key role in the process of plant infection. Aal could, via production of diols, use sEH in interactions (i.e. competition) with other microorganisms. Fungi, like other organisms, are in continuous contact with epoxides, which are ubiquitous and occur naturally in industrial settings, in the environment and in biochemical pathways. Some of them are highly toxic [35]. In contrast to animals, fungi do not have an effective excretion pathway and sEH would be the ideal enzyme to metabolize such reactive compounds, including a possible intermediate in the AAL toxin biosynthesis.

We are now in the process of purifying the enzyme. Working with pure enzyme will facilitate metabolic studies and will help to get a better understanding of its significance. The purification will also lead to a molecular biology work. Cloning of the enzyme will

Table 2. Specific activity of sEH *Alternaria alternata* f. sp. *lycopersici* with different substrates. Results are mean of three independent experiments performed in triplicate

Substrate	Specific activity (nmol/min/mg)
 *trans*-stilbene oxide (TSO)	0.34 ± 0.03
 *cis*-stilbene oxide (CSO)	0.16 ± 0.04
 *trans*-diphenylpropane oxide (tDPPO)	3.99 ± 0.13
 *cis*-diphenylpropane oxide (cDPPO)	3.29 ± 0.15
 9,10-epoxystearic acid	27.8 ± 1.0
 methyl-9,10-epoxystearate	2.9 ± 0.3

indicate how the fungal EH that we studied is related to EHs previously cloned from other organisms [22, 30].

Acknowledgements

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