

## Cress and Potato Soluble Epoxide Hydrolases: Purification, Biochemical Characterization, and Comparison to Mammalian Enzymes

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**Affinity chromatographic methods were developed for the one-step purification to homogeneity of recombinant soluble epoxide hydrolases (sEHs) from cress and potato. The enzymes are monomeric, with masses of 36 and 39 kDa and pI values of 4.5 and 5.0, respectively. In spite of a large difference in sequence, the two plant enzymes have properties of inhibition and substrate selectivity which differ only slightly from mammalian sEHs. Whereas mammalian sEHs are highly selective for *trans*- versus *cis*-substituted stilbene oxide and 1,3-diphenylpropene oxide (DPPO), plant sEHs exhibit far greater selectivity for *trans*- versus *cis*-stilbene oxide, but little to no selectivity for DPPO isomers. The isolation of a covalently linked plant sEH-substrate complex indicated that the plant and mammalian sEHs have a similar mechanism of action. We hypothesize an *in vivo* role for plant sEH in cutin biosynthesis, based on relatively high plant sEH activity on epoxystearate to form a cutin precursor, 9,10-dihydroxystearate. Plant sEHs display a high thermal stability relative to mammalian sEHs. This stability and their high enantioselectivity for a single substrate suggest that their potential as biocatalysts for the preparation of enantiopure epoxides should be evaluated.** © 2000 Academic Press

**Key Words:** epoxide hydrolase; epoxy fatty acid; affinity purification; mechanism, cutin biosynthesis.

Epoxide hydrolases (EH,<sup>5</sup> EC 3.3.2.3) catalyze the hydrolysis of epoxides or arene oxides to their corresponding diols by the addition of water (1). Mammalian hepatic microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH) have broad and complementary substrate selectivity (2). These EHs are known to detoxify mutagenic, toxic, and carcinogenic xenobiotic epoxides (3). The sEH is also involved in the metabolism of oxylipins, including epoxides of arachidonic and linoleic acids (4, 5), which are thought to be endogenous chemical mediators of vascular permeability (6). These epoxides of olefinic lipids have been found in association with physiological dysfunctions such as inflammation, hypoxia (7), and hypertension (8). Interestingly, numerous plant seeds are known to contain similar oxylipins (9) such as vernolic acid, a monoepoxide of linoleic acid. Their hydrolysis to the corresponding diol by epoxide hydrolases results in important intermediates for cutin synthesis (10–12), for the production of aromatic components (13), or for the antifungal defense of the plant (14).

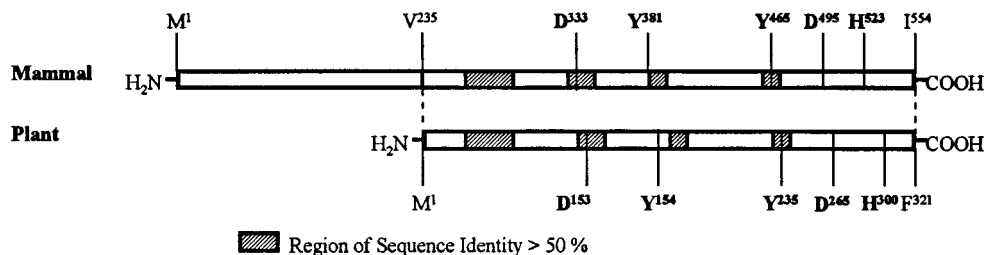
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<sup>5</sup> Abbreviations used: EH, epoxide hydrolase; mEH, microsomal epoxide hydrolase; sEH, soluble epoxide hydrolase; PsEH, potato sEH; CsEH, cress sEH; MseEH, mouse sEH; HseEH, human sEH; RseEH, rat sEH; AcNPV, *A. californica* nuclear polyhedrosis virus; BSA, bovine serum albumin; tDPPO, [<sup>3</sup>H]*trans*-1,3-diphenylpropene oxide; cDPPO, [<sup>3</sup>H]*cis*-1,3-diphenylpropene oxide; tSO, [<sup>3</sup>H]*trans*-stilbene oxide; cSO, [<sup>3</sup>H]*cis*-stilbene oxide; R-NEPC, (2*R*,3*R*)-4-nitrophenyl 2,3-epoxy-3-phenylpropyl carbonate; S-NEPC, (2*S*,3*S*)-4-nitrophenyl 2,3-epoxy-3-phenylpropyl carbonate; JH-III, juvenile hormone-III; ESA, epoxystearic acid; EODM, [<sup>14</sup>C]*cis*-9,10-epoxy-12-octadecenoate methyl ester.



**FIG. 1.** Comparison of mammal and plant sEH protein sequences. The displayed amino acid numbers correspond to the mouse and cress sEHs. The labeled residues include the N-terminal methionines, the C-terminals, the first residue of the mammalian C-terminal domain (V<sup>235</sup>), and five known catalytic residues (in bold). Vertical bars indicate the relative position of each amino acid on the protein linear sequence. The scheme was adapted from data published by Beetham *et al.* (21).

In comparison with animals, little is known about epoxide hydrolases of plants. Fatty acid epoxide hydrolases have been described from apple fruit skin (10), several plant seeds (15, 16), and whole rice plants (14) and seem ubiquitous in plants (16). However, only the soybean soluble EH was isolated and characterized (15, 17). In collaboration with other laboratories, cDNA encoding sEH of potato (PsEH) and cress (CsEH) was isolated and cloned (18, 19), and recently a putative sEH cDNA was cloned from tobacco by Gou *et al.* (20). These plant genes code for proteins 30% shorter on the N-terminus than the mammalian sEH (Fig. 1), but they display significant homology to the C-terminal domain of the mammalian sEH that contains the amino acid residues critical to catalytic activity (20, 21). Thus, a logical hypothesis is that plant EHs have a similar catalytic mechanism to the mammalian EHs (21), which transform their substrates in a two-step mechanism involving the formation and hydrolysis of a covalent hydroxyalkyl enzyme intermediate (2, 22, 23). However, this hypothesis has not yet been directly investigated.

A critical first step in understanding the catalytic mechanism, biochemistry, and biological role of an enzyme is its purification. An affinity purification procedure has been developed in the laboratory to purify large amounts of mammalian sEH easily and quickly (24, 25). This method uses benzylthiol-derivatized Sepharose to bind the enzyme and 4-fluorochalcone oxide for its elution. Unfortunately, this procedure gave poor results (extremely low yield and purity) for the plant enzymes (unpublished data). To develop an affinity method giving good recovery and purity, we first studied the inhibitory potency of several chalcone oxides to identify candidate molecules with potential utility in elution of plant sEHs from an affinity column. To identify candidate molecules for column derivation, we then investigated the binding capacity of several alkyl- and arylthiols coupled to Sepharose. Finally, we characterized the biochemical and enzymatic properties of affinity-purified cress and potato sEH in com-

parison with the mammalian sEH of mouse, human, and rat.

## MATERIALS AND METHODS

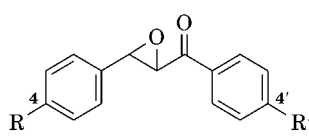
**Chemicals.** All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden). Chalcone oxides were previously synthesized in this laboratory (23).

**Synthesis of affinity matrix.** Affinity gels were synthesized following the method described by Wixtrom *et al.* (24). Sepharose CL-6B was washed extensively and successively with water, water/methanol (1:1), and 0.1 M NaOH. To 10 g of moist gel, 30 mg of NaBH<sub>4</sub>, and 2 ml of 1,4-butanediol diglycidyl ether was added 20 ml of 0.3 M NaOH. The mixture was swirled at room temperature overnight. The epoxy-activated gel was then sequentially and extensively washed with water, methanol/water (1:1), methanol, methanol/water (1:1), and water. The water was either freshly distilled or neutralized so that it was not acidic. Free epoxy functionality was assayed as described (25). A fivefold excess of thiol in 20 ml of methanol was added to the activated gel in 10 ml of 0.1 M NaHCO<sub>3</sub>. The gel was then gently swirled on a rotating table, to avoid fracturing the gel into fine particles. After mixing overnight, the derivatized Sepharose was washed extensively and successively with methanol/water (1:1), methanol, methanol/water (1:1), water, 0.5 M NaCl, water, 1 mM HCl, water, and ethanol/water (1:1). The resulting gel was stored at 4°C in absolute ethanol containing 0.1% butylated hydroxyanisole.

**Expression of recombinant sEH in the baculovirus system.** The cDNA of rat sEH from plasmid pUCcEH1 (26) was cloned into the (end-filled) *Bgl*II site of the baculovirus cotransfection plasmid pAcUW21 (27) to yield pAcUW21-RsEH. Recombinant *Autographica californica* nuclear polyhedrosis virus (AcNPV) was obtained by cotransfecting insect cells (line 21 from *Spodoptera frugiperda*) with this latter plasmid and linearized DNA (AcRP6) of AcNPV (28). The resulting purified plaque/virus (29) with the highest sEH activity was selected. A recombinant AcNPV containing the cDNA of cress sEH was constructed, using cress sEH cDNA from plasmid pEXcAtsEH1122 (19). Recombinant viruses of mouse, human, and potato sEH were previously constructed in the laboratory (18, 30, 31). The recombinant virus of rat sEH was kindly provided by Drs. M. Arand and F. Oesch of the University of Mainz, Germany (32).

Recombinant sEH was produced in High Five insect cell culture (derived from *Trichoplusia ni*; Invitrogen, San Diego, CA) infected at a multiplicity of infection of 0.1 virus per cell. The sEH enzyme activity was retained in the cells. After 90–96 h of incubation at 28°C, cells were harvested by centrifugation (100g for 10 min at 4°C). After resuspension in chilled sodium phosphate buffer (0.1 M, pH 7.4; Buffer A) containing 1 mM PMSF, EDTA, and DTT, cells

TABLE I

Inhibition of CsEH and PsEH by Racemic Substituted Chalcone Oxides, with Results for MsEH<sup>a</sup> for Comparison


Number	R	R'	IC <sub>50</sub> (μM) <sup>b</sup>		
			CsEH	PsEH	MsEH <sup>a</sup>
1	H	H	23 ± 1	3.4 ± 0.1	2.9 ± 0.3
2	F	H	4.7 ± 0.2	0.48 ± 0.02	1.3 ± 0.3
3	Br	H	2.2 ± 0.5	0.12 ± 0.04	0.7 ± 0.1
4	CH <sub>3</sub>	H	5.6 ± 0.2	0.16 ± 0.02	1.9 ± 0.2
5	CH <sub>3</sub> O	H	2.8 ± 0.7	0.19 ± 0.05	0.20 ± 0.02
6	NO <sub>2</sub>	H	11 ± 3	0.38 ± 0.05	1.8 ± 0.3
7	C <sub>6</sub> H <sub>5</sub>	H	>100	>100	0.14 ± 0.01
8	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	H	1.7 ± 0.3	>100	0.15 ± 0.01
9	H	F	22 ± 5	1.2 ± 0.4	1.8 ± 0.2
10	H	Br	13 ± 3	0.89 ± 0.02	0.6 ± 0.1
11	H	CH <sub>3</sub>	30 ± 5	1.4 ± 0.4	1.7 ± 0.2
12	H	CH <sub>3</sub> O	5.4 ± 0.2	0.21 ± 0.05	0.32 ± 0.04
13	H	NO <sub>2</sub>	16 ± 4	19 ± 2	1.5 ± 0.2
14	H	C <sub>6</sub> H <sub>5</sub>	>100	2.5 ± 0.6	1.37 ± 0.08
15	Br	CH <sub>3</sub> O	4.8 ± 0.6	0.21 ± 0.07	0.27 ± 0.05

<sup>a</sup> Results for MsEH are from Morisseau *et al.* (23).<sup>b</sup> Results are means ± standard deviation (*n* = 3).

were disrupted using a Polytron homogenizer (9000 rpm for 30 s). The homogenate was centrifuged at 12,000*g* for 20 min at 4°C. EH was purified from the supernatant. Protein concentration was quantified using the Pierce BCA assay (Pierce, Rockford, IL), using Fraction V bovine serum albumin (BSA) as the calibrating standard.

**Purification of recombinant sEH.** Recombinant mouse (MsEH), human (HsEH), and rat (RsEH) soluble epoxide hydrolases were purified from cell lysate by affinity purification as described by Wixtrom *et al.* (24). Purity of the enzymes was assessed by SDS-PAGE and electrofocusing gels as described below.

Recombinant cress sEH (CsEH) and potato sEH (PsEH) were purified on an analytical scale in glass tubes (14 × 100 mm) as follows. In a rotating tube, vacuum-dried gels (0.2 g) were washed twice with 5 ml of Buffer A (the gel was separated from the buffer by a quick centrifugation; 200*g* for 2 min). The gel was mixed with 5 ml of crude extract over 30 min at 4°C and then washed twice with 5 ml of buffer over 30 min. To elute the sEH, 5 ml of cold Buffer A containing 1 mM 4-bromo-4'-methoxychalcone oxide (compound **15** in Table I) and 1% DMF was mixed with the gel over 15 min. The unbound, washed, and eluted supernatants were stored between 16 and 20 h at 4°C prior to protein concentration and EH activity determinations.

For purification of the two plant enzymes on a preparative scale, 30 ml of gel (3,4-dichlorophenylthio- and phenylthio-Sephacrose for the cress and potato enzymes, respectively) was washed extensively with Buffer A and then mixed with 200 ml of supernatant from the described crude cell extract during 2 h at 4°C. The gel was washed three times with 300 ml of Buffer A containing 1% DMF. The EH was eluted with 200 ml of buffer containing 1 mM **15** and 1% DMF and collected in fractions of approximately 4 ml. The protein concentration and EH activity were tested the following day, and tubes containing activity were pooled and concentrated on Centricon-10 (Amicon, Beverly, MA).

**Enzyme assays.** Epoxide hydrolase activity was measured using racemic [<sup>3</sup>H]*trans*-1,3-diphenylpropene oxide (tDPPO, compound **16** in Table VI) as described previously (33). Briefly, 1 μl of a 5 mM

solution of [<sup>3</sup>H]tDPPO in DMF was added to 100 μl of enzyme preparation in Buffer A containing 0.1 mg/ml BSA ([S]<sub>final</sub> = 50 μM; ≈ 12,000 dpm/assay). The enzymes were incubated at 30°C for 5–10 min, and the reaction was quenched by addition of 60 μl of methanol and 200 μl of isooctane, which extracts the remaining epoxide from the aqueous phase. The activity was followed by measuring the quantity of radioactive diol formed in the aqueous phase using a liquid scintillation counter (Wallac Model 1409, Gaithersburg, MD). Assays were performed in triplicate. Kinetic constants were determined for tDPPO following the assay method described above, with substrate concentrations varying from 1.0 to 50.0 μM. *K*<sub>m</sub> and *k*<sub>cat</sub> were calculated from Lineweaver–Burk plots. EH activity was also measured using racemic [<sup>3</sup>H]*cis*-1,3-diphenylpropene oxide (**17**; cDPPO), [<sup>3</sup>H]*trans*-stilbene oxide (**18**; tSO), [<sup>3</sup>H]*cis*-stilbene oxide (**19**; cSO), and both enantiomers of 4-nitrophenyl 2,3-epoxy-3-phenylpropyl carbonate, (2*R*,3*R*) (**20**; R-NEPC) and (2*S*,3*S*) (**21**; S-NEPC) as described previously (3, 33, 34). EH activity using racemic 4-chlorophenyl glycidyl ether (**22**) as substrate was measured as follows. The enzyme was diluted in 100 μl of Buffer A and was incubated with 5 mM **22** for 5–10 min at 30°C. The reaction was stopped by addition of 100 ml of methanol containing 0.2 mM 1-(4-chlorophenoxy)propan-2-ol as internal standard. The quantity of diol formed was determined by HPLC analysis on a 100 Å Reliasil C18 (1 × 150 mm) column on a Microbore HPLC system (Michrom Biore-sources Inc., Auburn, CA). The mobile phase was water/acetonitrile (55:45), with a flow rate of 50 μl min<sup>-1</sup>. The compounds were detected by UV absorbance at 230 nm. The diol, external standard, and initial substrate have retention times of 12.3, 16.2, and 18.3 min, respectively. Racemic <sup>3</sup>H-labeled juvenile hormone-III (**23**; JH-III), <sup>14</sup>C-labeled *cis*-9,10-epoxystearic acid (**24**; ESA), and [<sup>14</sup>C]*cis*-9,10-epoxy-12-octadecenoate methyl ester (**25**; EODM) EH activities were measured as described (33, 35).

**Electrophoresis and molecular weight determination.** SDS-PAGE was conducted on 1-mm-thick slab gels consisting of 12% acrylamide resolving gel and 4% acrylamide stacking gel at pH 8.8 in the presence of 0.1% SDS (36). The samples were dissolved in Tris/

HCl loading buffer (62.5 mM, pH 8.8) containing 10 g/liter of SDS, 10% glycerol, and 2%  $\beta$ -mercaptoethanol and heated at 100°C for 2 min. Proteins were stained with 0.1% Coomassie brilliant blue R-250. Electrofocusing was performed with pH 3.0–7.0 gradient gels by using precast gels and standard procedures from Novex (San Diego, CA). The molecular weight associated with EH activity of the purified enzymes was estimated from the elution profile on a gel filtration column. The enzyme (0.5 ml) was applied to a Sephacryl S100 (Pharmacia, Uppsala, Sweden) column (1.5  $\times$  100 cm), equilibrated with Buffer A (flow rate, 10 ml/h; fraction volume, 1 ml). The molecular weight was calculated by comparing the elution of the EH activity with that of the following standard proteins: alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa). The void and exclusion volumes were determined by using Dextran Blue and vitamin B<sub>12</sub>.

**IC<sub>50</sub> assay conditions.** The IC<sub>50</sub> for each inhibitor was determined using tDPPPO as substrate ([S]<sub>final</sub> = 50  $\mu$ M). Crude extracts of CsEH or PsEH (100  $\mu$ l, 1 mU ml<sup>-1</sup>) were incubated 15 min at 30°C with 1  $\mu$ l of each inhibitor diluted in DMF ([I]<sub>final</sub> = 0.01–100  $\mu$ M) before testing the remaining activity as described. These conditions were chosen to better discriminate among the inhibitors. By definition, the IC<sub>50</sub> is the concentration of inhibitor that reduces enzyme activity by 50%. IC<sub>50</sub> was determined by regression of at least five datum points, with a minimum of two points in the linear region of the curve on either side of the IC<sub>50</sub>. The data were generated from at least three separate runs. In at least one run, inhibitors of similar potency were included to ensure rank order. Given that hydrolysis of the covalent chalcone oxide enzyme intermediate is the rate-limiting step in sEH inhibitor turnover (23), the time-dependent inhibitor clearance was measured, allowing a direct estimation of the half-life of the enzyme–inhibitor complex. The two plant enzymes were incubated with inhibitors **3**, **12**, or **15** at concentrations (5 and 0.1  $\mu$ M for CsEH and PsEH, respectively) which gave 60–80% of initial inhibition. Enzyme activity recovery was followed over time until approximately 90% of the initial activity was recovered.

**Analysis of enzyme–inhibitor adducts.** Recombinant cress soluble epoxide hydrolase (100  $\mu$ g) was suspended in 100  $\mu$ l of Buffer A and incubated at room temperature with or without 4-bromo-4'-methoxychalcone oxide (**15**) dissolved in 1  $\mu$ l of DMF ([I]<sub>final</sub> = 1 mM). After 10 s, the reaction was halted and the enzyme was precipitated by the addition of 100  $\mu$ l of 1% formic acid in methanol (v/v). Excess inhibitor was extracted with two 300- $\mu$ l chloroform washes. To remove buffer salts, the sample was diluted to 1.5 ml with double-distilled water. Under these conditions the protein precipitated and was recovered by centrifugation (10,000g for 5 min). The pellet was washed twice with water, and then the aggregated protein was suspended in 50  $\mu$ l of 1% formic acid in water. Modified protein and unmodified protein were diluted in an equal volume of sinapinic acid (Hewlett–Packard, San Jose, CA) and analyzed on a Voyager DE-STR Biospectrometer Workstation high-resolution MALDI-TOF (PerSeptive Biosystems, San Francisco, CA).

## RESULTS

**Enzyme inhibition.** IC<sub>50</sub> values provided relative inhibition potency of the tested chalcone oxides (Table I) and directed the selection of useful compounds for the elution of the two enzymes. While both enzymes displayed a similar pattern of inhibition, the IC<sub>50</sub> values for PsEH are overall about one order of magnitude smaller than for CsEH. Such difference could be explained by a different concentration of enzyme in the two crude extracts used, differential affinity, and varying turnover for the substrate used. It has been previously shown that for some chalcone oxides the observed

**TABLE II**  
Apparent Half-Lives of Enzyme–Inhibitor Intermediates at 30°C

Inhibitor	<i>t</i> <sub>1/2</sub> (min) <sup>a</sup>	
	CsEH <sup>b</sup>	PsEH <sup>b</sup>
<b>3</b>	26.7 $\pm$ 0.7	32 $\pm$ 1
<b>12</b>	27 $\pm$ 2	25 $\pm$ 1
<b>15</b>	54 $\pm$ 1	43 $\pm$ 3

<sup>a</sup> Results are means  $\pm$  standard deviation (*n* = 3).

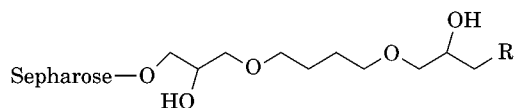
<sup>b</sup> [I] = 5  $\times$  10<sup>-6</sup> (CsEH) or 10<sup>-7</sup> (PsEH).

IC<sub>50</sub> is dependent upon the concentration of catalytic site, the substrate used, and the time of incubation because these inhibitors are actually substrates that are slowly turned over (23). Compared to the mammalian enzymes (23, 37), the plant enzymes displayed a very different pattern of inhibition; i.e., the 4-phenylchalcone oxide **7** is an excellent mammalian sEH inhibitor, while it displayed no inhibition of the cress and potato enzymes (Table I). Of the seven substituents tested at position 4 (**2–8**), the best inhibitory potencies (smallest IC<sub>50</sub>) were obtained for the bromo derivative **3**. For the 4' position (compounds **9–14**), the best inhibition was obtained for the methoxy derivative **12**. The inhibitory potency of double-substituted derivative **15** is indistinguishable from that of **12**. From the set of compounds tested, unlike for the mammalian enzymes (23), no significant structural relationship was found for either plant sEH.

We then investigated the half-life of three potent inhibitors **3**, **12**, and **15** in the presence of the plant enzymes (Table II). For the mammalian enzymes, chalcone oxides have been shown to be weak substrates that form a covalent complex, whose hydrolysis is rate limiting in the sEH catalytic cycle (23). Therefore measurements of inhibitor half-life can be utilized to estimate the enzyme–inhibitor complex turnover. For **3**, CsEH and PsEH formed a more stable covalent intermediate than the mouse sEH (MsEH *t*<sub>1/2</sub> = 4.2 min). For both plant sEHs, the most stable inhibition was obtained with compound **15**, and this compound was chosen as the eluting inhibitor for the purification procedure. Incubation of the inhibited enzyme overnight at 4°C allowed a total recovery of the enzymatic activity. The noninhibitory products obtained are readily removed by dialysis.

**Optimization of the affinity gel.** The binding capacity and enzyme retention of an affinity gel are respectively determined by the gel ligand density and the ligand specificity for the enzyme of interest. If either parameter is too great, however, elution of the target enzyme can be compromised. To optimize the specificity of the ligand and to avoid elution problems, all gels

**TABLE III**  
Analytical-Scale Purification of CsEH and PsEH on the Affinity Matrix



R	Gel	Percentage recovered of total activity					
		CsEH			PsEH		
		Unbound	Washed	Eluted	Unbound	Washed	Eluted
	<b>A</b>	76	9	1	83	12	0
	<b>B</b>	71	10	1	79	13	0
	<b>C</b>	54	9	25	64	18	3
$n\text{-C}_8\text{H}_{17}\text{-S}$	<b>D</b>	34	7	33	44	13	20
$n\text{-C}_{10}\text{H}_{21}\text{-S}$	<b>E</b>	70	12	5	69	17	3
	<b>F</b>	52	1	25	6	2	43
	<b>G</b>	67	12	3	6	3	48
	<b>H</b>	74	11	5	76	12	4
	<b>I</b>	20	5	49	7	5	41
	<b>J</b>	21	6	42	19	13	34
	<b>K</b>	16	5	46	18	12	40
	<b>L</b>	19	6	48	23	11	37
	<b>M</b>	14	3	53	12	9	42
	<b>N</b>	74	11	1	76	12	3

<sup>a</sup> Elution with compound **2** (purification method classically used for mammal sEHs; 24).

were synthesized with a ligand density of  $\sim 10 \mu\text{mol/g}$  of wet gel. The binding properties of 13 alkyl- and arylthiols coupled to epoxy-activated Sepharose CL-6B were tested at an analytical scale for the two plant

enzymes (Table III). Of the five alkylthio-derived Sepharoses (**A–E**), the best binding (minimum activity in unbound and washed fractions) was obtained for the octylthioether gel **D**. However, 40 and 60% of the cress

TABLE IV  
Preparative-Scale Purification

	Total activity (U) <sup>a</sup>	Yield (%)	Total protein (mg)	Specific activity (mU/mg)	Coefficient of purification
CsEH					
Crude extract	292	100	1740	167	1
Unbound	187	64	1060	177	1.1
Active fraction	93	32	24	3910	23.4
PsEH					
Crude extract	138	100	1140	121	1
Unbound	94	68	1280	74	0.6
Active fraction	37	27	14	2670	22.1

<sup>a</sup> 1 U is defined as 1  $\mu$ mol of diol formed per minute.

and potato activities, respectively, failed to bind with this gel.

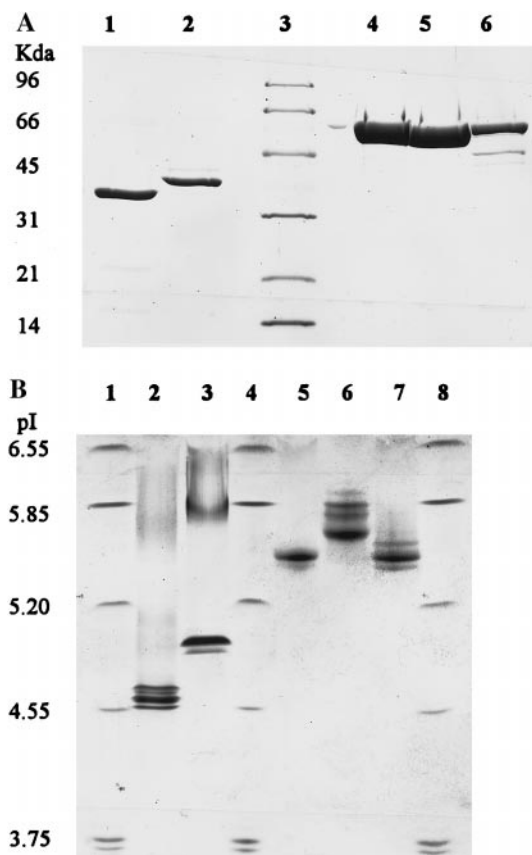
We then investigated the efficiency of a single cycloalkylthio-derived (**F**) and seven arylthio-derived (**G–M**) Sepharose gels. The cress enzyme was efficiently bound (>70% of initial activity) by gels containing a halogenated ring (**I–M**). Moreover, 40–50% of the initial activity (60–70% of bound activity) could be eluted from these latter gels using compound **15**. Of these five gels, the purest enzyme was obtained using 3,4-dichlorophenylthio-Sepharose (**L**), as judged by the coefficient of purification (17 for **L** versus 10–12 for the four others) and SDS–PAGE (results not shown). Therefore, **L** was chosen for the purification of the cress enzyme. Unlike the cress, the potato enzyme was well bound (>90%) to gels containing a cyclohexyl (**F**) or phenyl (**G**) moiety adjacent to the sulfur atom. Adding a methylene between the ring and the sulfur (**H**) resulted in a 10-fold decrease in binding. Interestingly, the same addition of a methylene dramatically increased binding with the mammalian sEH (24). Relative to gel **F**, halogenating the phenyl ring at position 4 (**J–L**) resulted in a 3-fold decrease in bound enzyme, while halogenation of position 3 had no (**I**) or little (**M**) effect on gel binding properties. Gel **G** yielded more purified enzyme ( $\approx$ 50% of initial activity) than the three other gels (**F**, **I**, and **M**) while these four gels gave similar coefficients of purification ( $\approx$ 20). Therefore, **G** was chosen for the affinity purification of the potato enzyme. Interestingly, the data for gel **N**, developed for mammalian sEH purification (24), indicated that our past difficulty in purifying plant EHs using the mammalian purification reagents was due to both poor binding ( $\approx$ 10%) and inefficient elution (10–30% of bound activity).

**Enzyme purification.** The recombinant plant enzymes were purified in large scale from 2 liters of cell culture. Crude extracts were prepared from cells harvested at 96 h after viral infection, as described under Materials and Methods. Results of purification utiliz-

ing gels **L** and **G** for recombinant CsEH and PsEH, respectively, are displayed in Table IV. For both enzymes, a large quantity of enzyme was unbound: 64 and 68% for the cress and potato sEHs, respectively, suggesting the gels may have been overloaded. The binding capacity of these gels was estimated at approximately 1 mg of pure enzyme per milliliter of gel. The elution yields of 90 and 84% obtained for the CsEH and PsEH, respectively, are superior to the 64 and 53% yields obtained on the analytical scale. For both enzymes, a single-step purification factor of  $\approx$ 20 was obtained, indicating that the recombinant enzymes represent approximately 5% of the protein produced by the infected insect cells. Such a value has been commonly obtained in the baculovirus expression system (29). Recombinant sEHs from mouse, human, and rat were purified by affinity chromatography (24) from insect cell cultures infected with the corresponding recombinant baculoviruses (30, 31). These enzymes were used for comparison with the two plant enzymes.

**Purity and structural characterization.** The purity of the five enzymes was assessed by denaturing and isoelectric focusing electrophoresis (Fig. 2). Coomassie blue staining of purified enzymes separated by SDS–PAGE showed a single major band for all enzymes except for the rat enzyme, which was slightly contaminated by a band at 50 kDa (Fig. 2A). Minor bands were observed in plant enzymes. Scanning densitometry indicated a purity of 92% for the rat enzyme, while the four other enzymes had purities of at least 97%. However, for each enzyme several bands were obtained on isoelectric focusing gels (Fig. 2B). Such results have been previously observed for mammalian sEHs from both natural and recombinant sources (38).

As expected (3), the three mammalian sEHs have denatured masses of approximately 60 kDa and *pI* values of  $\sim$ 5.5, while the cress and potato sEHs have denatured masses of 36 and 39 kDa and *pI* values of 4.5 and 5.0, respectively. Mass determination using a gel filtration column indicated that the mammalian



**FIG. 2.** Electrophoresis analysis. (A) SDS-PAGE. Lane 1, 10  $\mu\text{g}$  of cress sEH; lane 2, 10  $\mu\text{g}$  of potato sEH; lane 3, molecular weight markers; lane 4,  $\sim 25$   $\mu\text{g}$  of mouse sEH; lane 5,  $\sim 25$   $\mu\text{g}$  of human sEH; lane 6, 10  $\mu\text{g}$  of rat sEH. (B) Isoelectric focusing gel (10  $\mu\text{g}$  of protein by lane). Lanes 1, 4, and 8, standard proteins; lane 2, cress sEH; lane 3, potato sEH; lane 5, mouse sEH; lane 6, human sEH; lane 7, rat sEH.

enzymes have native mass of  $\sim 130$  kDa while the plant enzymes have native mass of  $\sim 40$  kDa. These results indicated that the recombinant mammalian sEHs are

dimeric, as found before for sEHs directly purified from mammalian tissues (3), while the plant sEHs characterized here are monomeric under the conditions of the gel permeation used.

**Enzyme stability.** The stability of the five enzymes was investigated for temperatures ranging from 0 to 60°C. The results are displayed in Table V. The mammalian sEHs appear less stable than the plant enzymes. The former enzymes, even at 37°C, their natural temperature of action, have relatively short lives ( $t_{1/2} < 2$  h), which could be associated with enzyme regulation *in vivo*. For the plant enzymes, the potato sEH appeared more stable than the cress sEH. Such temperature stability may also reflect the *in vivo* role of these enzymes.

**Substrate selectivity.** The activity of the five recombinant enzymes was tested toward ten substrates available in the laboratory: seven benzyl- or phenyl-substituted epoxides (compounds **16**–**22**) and three natural lipid epoxides (compounds **23**–**25**). The rates of hydration of compound **16** are shown in Table VI as  $K_M$ ,  $k_{\text{cat}}$ , and specific activity, while the rates of hydration of compounds **17**–**25** are shown as percentages relative to the specific activity of **16**. Over all the substrates tested, the three mammalian enzymes gave similar results while the two plant EHs differed somewhat from each other and from the mammalian enzymes.

Compound **16** is a surrogate substrate developed for the MsEH (33). All five enzymes display similar  $K_M$  values for this racemic substrate (5–7  $\mu\text{M}$ ). However, the two plant enzymes, especially CsEH, have smaller  $k_{\text{cat}}$  values than the rodent enzymes (MsEH and RsEH). As expected (33), the mammalian EHs display a strong preference for *trans*-substituted epoxides when we compare **16** and **18** to their *cis*-isomers **17** and **19**, respectively (Tables VI and VII). Moreover, the presence of one extra methylene between the epoxide

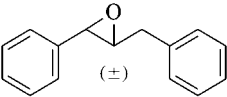
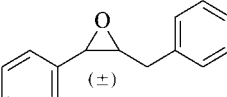
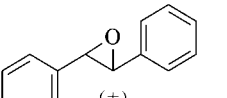
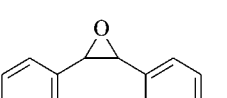
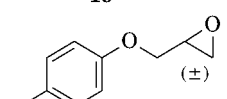
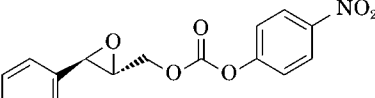
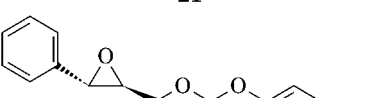
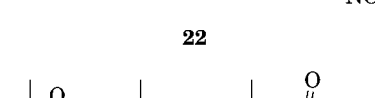
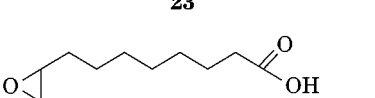
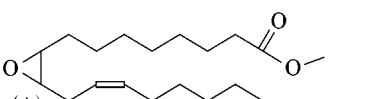
**TABLE V**  
Temperature Effect on Enzyme Stability

Temperature (°C)	$t_{1/2}$ (h)				
	MsEH	HsEH	RsEH	CsEH	PsEH
0	>24 (8) <sup>a</sup>	>24 (17) <sup>a</sup>	>24 (4) <sup>a</sup>	>24 (<2) <sup>a</sup>	>24 (<2) <sup>a</sup>
30	17.4 $\pm$ 0.2	8.1 $\pm$ 0.4	20.3 $\pm$ 0.5	>24 (<2) <sup>a</sup>	>24 (<2) <sup>a</sup>
37	1.8 $\pm$ 0.2	1.3 $\pm$ 0.1	2.0 $\pm$ 0.1	>24 (12) <sup>a</sup>	>24 (<2) <sup>a</sup>
45	<0.1	<0.1	<0.1	10.2 $\pm$ 0.7	>24 (24) <sup>a</sup>
51	nd <sup>b</sup>	nd	nd	6.4 $\pm$ 0.1	>24 (41) <sup>a</sup>
55	nd	nd	nd	0.93 $\pm$ 0.08	4.7 $\pm$ 0.3
59	nd	nd	nd	0.38 $\pm$ 0.02	0.87 $\pm$ 0.02

<sup>a</sup> The number in parentheses is the percentage of activity lost after 24 h of incubation at the indicated temperature.

<sup>b</sup> nd, not determined.

**TABLE VI**  
Substrate Selectivity of the Purified sEHs

Substrate		MsEH	HsEH	RsEH	CsEH	PsEH
 <b>16</b>	$K_M(\mu\text{M})$ $k_{\text{cat}}(\text{s}^{-1})$  Activity ( $\text{mUmg}^{-1}$ ) <sup>a</sup>	$4.3 \pm 0.6$ $18.0 \pm 0.3$  $17,000 \pm 300$	$6.2 \pm 0.6$ $4.3 \pm 0.3$  $4,500 \pm 200$	$6.9 \pm 0.6$ $12.0 \pm 0.4$  $10,200 \pm 700$	$7.0 \pm 0.3$ $1.46 \pm 0.02$  $2,490 \pm 90$	$8.3 \pm 0.2$ $4.76 \pm 0.03$  $7,500 \pm 400$
Relative activity <sup>b</sup>						
 <b>17</b>		$3.0 \pm 0.1$	$18.1 \pm 0.7$	$2.1 \pm 0.1$	$99 \pm 4$	$44 \pm 2$
 <b>18</b>		$2.8 \pm 0.1$	$1.2 \pm 0.1$	$1.0 \pm 0.1$	$84 \pm 2$	$5.6 \pm 0.2$
 <b>19</b>		$0.4 \pm 0.1$	$0.21 \pm 0.06$	$0.08 \pm 0.01$	$0.2 \pm 0.1$	$0.06 \pm 0.01$
 <b>20</b>		$5.3 \pm 0.1$	$14.6 \pm 0.1$	$5.1 \pm 0.1$	$7.6 \pm 0.1$	$0.83 \pm 0.01$
 <b>21</b>		nd <sup>c</sup>	nd	nd	$38 \pm 3$	<0.01
 <b>22</b>		nd	nd	nd	$15.2 \pm 0.2$	$34 \pm 1$
 <b>23</b>		$6.3 \pm 0.9$	$6.3 \pm 0.9$	$3.5 \pm 0.4$	$11.9 \pm 0.8$	$7.1 \pm 0.7$
 <b>24</b>		$6.7 \pm 0.2$	$7.9 \pm 0.3$	$4.1 \pm 0.1$	$30 \pm 1$	$13.4 \pm 0.9$
 <b>25</b>		$6.5 \pm 0.7$	$5.2 \pm 0.4$	$3.5 \pm 0.3$	$59 \pm 3$	$12 \pm 2$

Note. Results are means  $\pm$  standard deviation ( $n = 3$ ).

<sup>a</sup> 1 U is defined as 1  $\mu\text{mol}$  of diol formed per minute.

<sup>b</sup> Expressed as percentage of **16** specific activity.

<sup>c</sup> nd, not determined.



TABLE VII

Preference of Purified sEH for *trans* vs *cis* Isomers

	Ratio of ( <i>trans</i> isomer)/( <i>cis</i> isomer) activity <sup>a</sup>				
	MseEH	HseEH	RseEH	CseEH	PseEH
<b>16/17</b>	33	5.5	47	1.0	2.3
<b>18/19</b>	7	5.7	13	420	93
<b>21/22</b>	1.3 <sup>b</sup>			2.5	>3400 <sup>c</sup>

<sup>a</sup> Ratio calculated from data in Table VI.<sup>b</sup> Data from Dietze *et al.* (34).<sup>c</sup> Ratio of **22/21**.

ring and one phenyl ring of compounds **16** and **17** allows a 10- to 100-fold faster hydrolysis by the mammalian sEH than for the corresponding less flexible stilbene oxides (**18** and **19**, respectively). For the plant enzymes, the same trend in *trans* preference was observed; however, the relative *cis/trans* preference was more dramatic for the tested stilbenes (Tables VI and VII). For CseEH, no difference in activity is observed for *trans*- and *cis*-DPPO (**16** and **17**), while the *trans*-stilbene oxide **18** is hydrolyzed 420-fold faster than its *cis* isomer **19**; moreover, the two *trans* epoxides studied (**16** and **18**) are transformed with similar speed. For PseEH, as for the mammalian enzymes, a big difference in activity is observed between the presence (**16** and **17**) or the absence (**18** and **19**) of an extra methylene between the epoxide ring and one phenyl ring. However, like CseEH, PseEH hydrolyzed *trans*-stilbene oxide **18** 100-fold faster than its *cis* isomer **19**, while only a 2-fold difference was observed for the *trans*- and *cis*-DPPO (**16** and **17**).

The monosubstituted epoxide **20** was hydrolyzed at a reasonable rate by the mammalian enzymes. However, it is relatively poorly transformed by the two plant sEHs. Chemically, one would expect higher turnover of such a terminal epoxide compared to more hindered compounds. Hydrolysis of the two enantiomers (**21** and **22**) of spectrophotometric substrate NEPC (34) was assayed only with the plant sEHs. The cress enzyme hydrolyzed preferentially the (2*R*,3*R*) enantiomer **21** ~2.5 faster than its optical isomer, while the potato sEH hydrolyzed only the (2*S*,3*S*) enantiomer **22**. Interestingly, MseEH hydrolyzes the two enantiomers of NEPC (**21** and **22**) with similar activity (34). The three mammalian sEHs hydrolyzed the three lipid epoxides tested (**23**–**25**) with similar relative activities. The two plant sEHs are 2- to 5-fold less active toward the terpenoid epoxide **23** than for the epoxy fatty acid (**24** and **25**). CseEH is 2-fold more active on the linoleate mono-epoxide **25** than the epoxy stearate **24**, while PseEH has similar activities for both of these compounds.

*Identification of enzyme–substrate covalent intermediate.* Based on sequence analysis, the plant sEHs are hypothesized to have a catalytic mechanism similar to that of the mammalian sEHs (21), involving a covalent enzyme–substrate intermediate. To provide structural support for this hypothesis, we isolated and characterized an enzyme–substrate intermediate using plant cress sEH. Because chalcone oxides are sEH substrates with low turnover (23), we incubated purified CseEH with an excess of **15**. After a few seconds, the enzyme was precipitated (see Materials and Methods). We performed a mass spectral analysis of the resulting denatured protein with or without **15** preexposure (Fig. 3). Positive-mode analysis of the native protein (Fig. 3A) yielded an observed mass of 36,273 Da, which is 150 Da less than the predicted theoretical mass determined from the genetic sequence of the cDNA, 36,423 Da (19). This result is consistent with the loss of the N-terminal methionine residue. In addition, a series of multiplets are observed in this spectrum separated by ~220 Da and correspond to nonspecific adducts with the employed laser absorption matrix, sinapinic acid (*m/z* 224). Due to the low protein concentrations used for this analysis (~30 fmol/analysis), observation of adducts with the laser absorption matrix are not unexpected. Incubation of the enzyme with **15** (Fig. 3B) produced a molecular weight shift of 333–334 Da in ~80% of the enzyme, which corresponds well with the mass of **15** (~1:1 333:335 Da). In addition, we again see apparent sinapinic acid adducts with the modified protein (i.e., [M + H + ~220]<sup>+</sup>) but do not observe [M + H + 2(**15**)]<sup>+</sup> ions, further supporting the selective covalent nature of the interaction of **15** with CseEH. All discussed masses are within 0.005% of theoretical.

## DISCUSSION

This report describes the purification and characterization of recombinant sEH from two plants, cress and potato, and their comparison with mammalian sEH. The results obtained clearly show the utility and effectiveness of new affinity gels/methods allowing one-step preparation of these two plant sEHs. Elution yields were over 80%, and the proteins were highly pure as judged by SDS–PAGE (Fig. 1A). It is likely that a similar approach of hydrophobic binding coupled with specific elution could be applied to many proteins with a lipophilic binding pocket. Although unnecessary in the affinity purifications described herein, inclusion of a mild detergent in one or more of the washing steps can reduce contamination of the target enzyme by other proteins (24). For ligands with high hydrophobicity or high loading capacity, low levels of detergent in the eluting buffer also can increase yield. The multiple bands observed on electrofocusing gel (Fig. 1B) could be due to differential secondary posttranslational modifi-

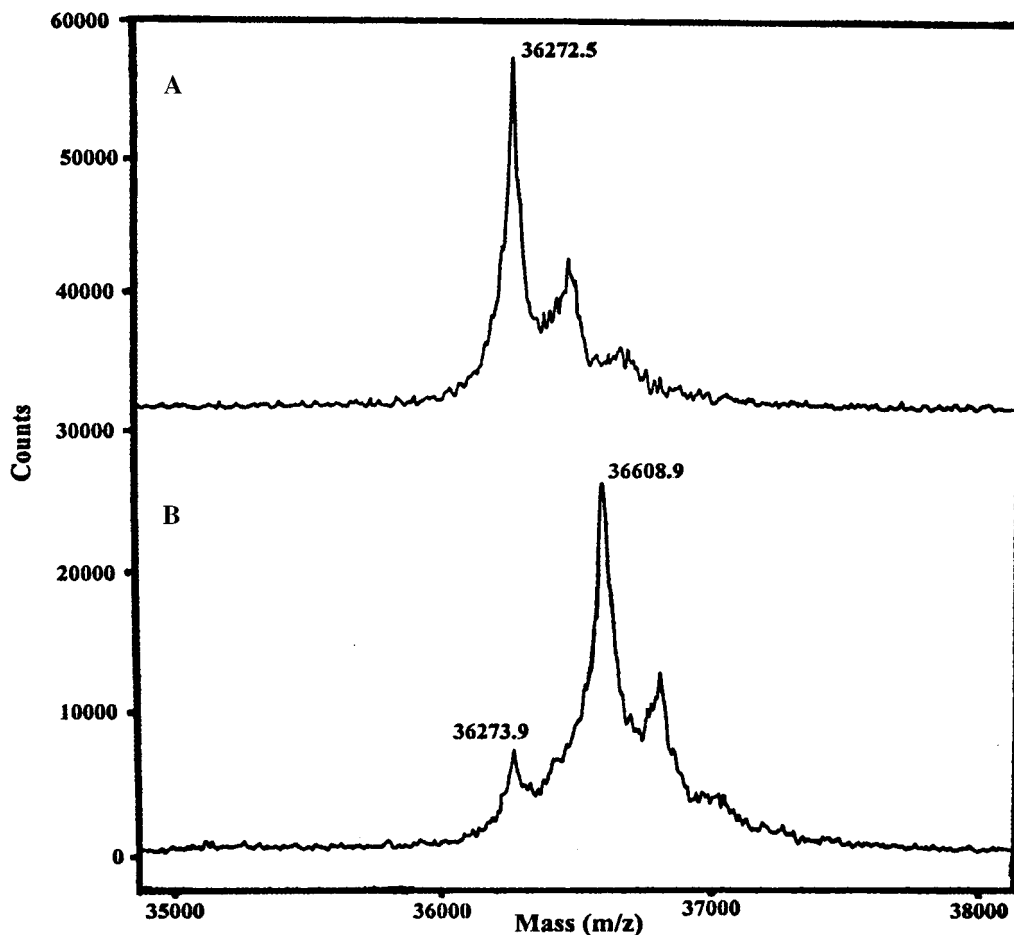


FIG. 3. High-resolution MALDI-TOF analysis of the purified cress sEH in the absence (A) or in presence (B) of the inhibitor 15.

cations, such as slight variations at the N-terminus, glycosylation, or phosphorylation, which sometimes occur when expressing recombinant proteins in the baculovirus system (29). We failed to detect evidence of glycosylation (results not shown). An alternative explanation, that the multiple bands are artifacts due to the particular IEF gels used, is unlikely since similar results were obtained when mammalian sEHs were separated on IEF gels from different vendors utilizing different ampholines (38).

As displayed in Fig. 1, sequence alignments show these plant sEH genes to be 30% shorter than the corresponding mammalian genes and, in fact, are missing the N-terminal domain of the mammalian enzymes (21). Recently, the determination of the crystal structure of the murine sEH (22) showed that this N-terminal domain is likely involved in the formation and stabilization of a homodimer. The two plant EHs studied here were found to be monomeric, supporting such a role for the N-terminal domain of mammalian sEH. The previously purified plant sEH from soybean was reported to be dimeric (15).

Despite low sequence homology, the plant and mammalian sEHs display an overall structural identity in their C-terminal domains (21), suggesting these sEHs are structurally and mechanistically similar. However, the selectivities of both plant enzymes toward the chalcone oxides (Table I) and a series of substrates (Table VI) are quite different from the selectivity to the same substrate of the mammalian sEHs (22, 33). As shown in Table VII, there is less *trans/cis* selectivity for the plant enzymes than for the mammalian sEH (especially for the two rodent enzymes) with compound **16** versus **17**, but a far greater *trans/cis* discrimination for compound **18** versus **19**. Since the only difference between **16/17** and **18/19** is due to the presence of an extra methylene group in **16/17**, the data suggest there is steric hindrance between the *cis*-**19** compound and the plant enzymes at the region of the biocatalysis that interacts with the epoxy group. Additionally, the mammalian enzymes have a similar hindrance, except it is located more distal to the enzyme region that interacts with the epoxide functionality, as indicated by mammalian sEH selectivity against compound **17** versus

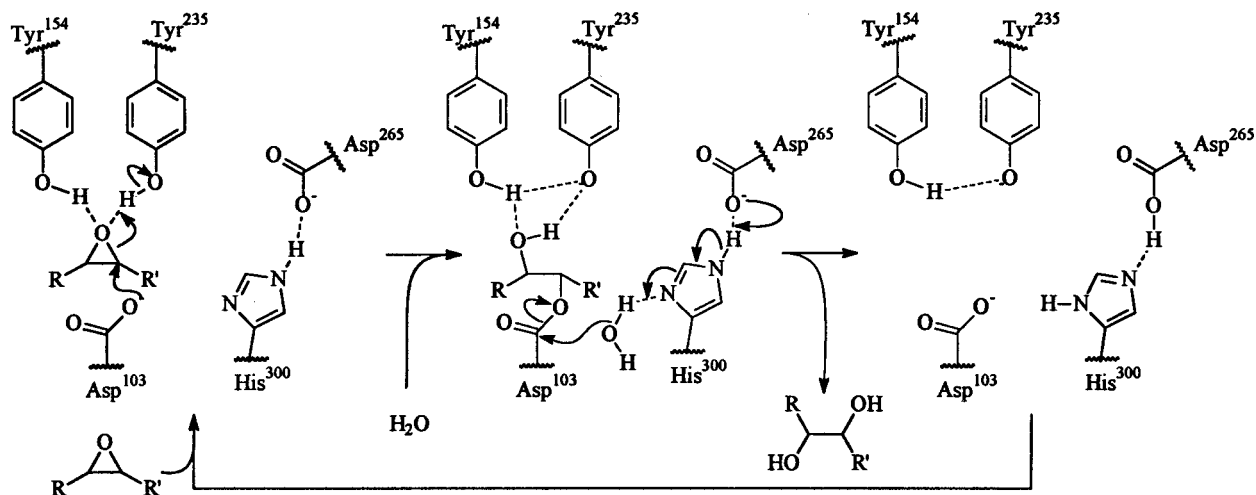


FIG. 4. Proposed mechanism for the plant sEH. The amino acid residue numbers correspond to the cress enzyme.

**16.** This suggests that, while similar, the active center of the plant and mammalian sEHs have different orientations relative to their respective hydrophobic substrate binding pockets. One plausible generalization is that relative to the mammalian sEH (22), the plant sEH hydrophobic pockets are restricted proximal to the epoxy-interacting regions but are "wider" as one moves away from the site of catalysis. Moreover, because both plant sEHs have relatively little activity on the monosubstituted epoxide **20** compared to di- or trisubstituted compounds, except for **19** (Table VI), the enzymes may require two substitutions on each side of the epoxide moiety to properly align the substrate. Such preference for di- over monosubstituted epoxides was observed for the cytosolic EH from mouse liver (3, 39). A high and opposite enantioselectivity was observed for the two plant sEHs on compounds **21** and **22**, suggesting that these enzymes should be further investigated as potential biocatalysts for the synthesis of fine organic chemicals (40).

While these enzymatic results suggest structural variance between plant and mammalian sEHs, the characterization of a covalent intermediate between CseEH and compound **15** (Fig. 3) provides evidence that the catalytic mechanism described for the mammalian EH (22) is functionally conserved in plant EH. By analogy with mammalian EH (21), Asp<sup>103</sup> and Asp<sup>105</sup> are the catalytic residues of the cress and potato EHs, respectively, which attack the epoxide ring to form a hydroxy-alkyl ester intermediate (Fig. 4). Such attack is facilitated by the polarization of the epoxide (23). For both the cress and the potato sEHs, Tyr<sup>235</sup> corresponds to Tyr<sup>465</sup> of the murine enzyme and likely plays the role of general-acid catalyst in activating the epoxide ring (22). The MseEH Tyr<sup>381</sup> is proposed to have this role also (22). Referring to the published amino acid sequence alignment (21), in removal of the proposed gap in the

mammalian sequence between Tyr<sup>384</sup> and Phe<sup>385</sup>, Tyr<sup>154</sup> from both the potato and cress enzymes directly corresponds to the mammalian Tyr<sup>381</sup>. For the murine enzyme, the covalent intermediate is hydrolyzed by a molecule of water activated by the Asp<sup>495</sup>-His<sup>523</sup> pair (22). By analogy of sequence (21), the Asp<sup>265</sup>-His<sup>300</sup> pair should activate the catalytic water for both CseEH and PseEH.

Finally, results obtained clearly show that both plant EHs are very active on epoxy fatty acids, an activity that likely relates to the biological role(s) of sEH in plants. Dihydroxystearic acid is an important intermediate for the formation of cutin (41), which covers and protects the aerial parts of plants, suggesting a role of sEH in cutin synthesis (10–12). In cress and potato, EHs are mainly expressed in the aerial vegetative part of the plants (18, 19), in support of this hypothesis. These tissues are exposed to sunlight and thus exposed to relatively high temperature. Such "natural" high temperatures could explain the thermal stability observed, especially for the potato enzyme. Alternatively, diols from monoepoxide of linoleic acid are toxic to numerous cell lines (5) and thus could participate in the plant defense as proposed (14, 20). In a different way, EH could be implied in the metabolism of jasmonic acid, a phytohormone important in plant pathogen defense (42). Inhibition of EH activity in plants could be a way to investigate the biological importance of the enzyme. New EH inhibitors recently reported (43), such as 1,3-dicyclohexylurea, which gives IC<sub>50</sub> values of 0.5 and 0.02 μM for PseEH and CseEH, respectively, will certainly aid such investigations.

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