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Occurrence of fatty acid epoxide hydrolases in soybean (*Glycine max*)

Purification and characterization of the soluble form

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Epoxide hydrolases catalysing the hydration of *cis*-9,10-epoxystearate into *threo*-9,10-dihydroxystearate have been detected in soybean (*Glycine max*) seedlings. The major activity was found in the cytosol, a minor fraction being strongly associated with microsomes. The soluble enzyme, which was purified to apparent homogeneity by $(NH_4)_2SO_4$ fractionation, hydrophobic, DEAE- and gel-filtration chromatographies, has a molecular mass of 64 kDa and a pI of 5.4.

INTRODUCTION

Epoxidized fatty acids, particularly from the C_{18} family, are naturally occurring metabolites in higher plants [1]. They are considered, along with their dihydroxy derivatives, obtained by hydrolysis of the oxirane, to be involved in the host's defence against fungal and bacterial pathogens [2]. Cutin was also found to contain high levels of such epoxy fatty acids and, importantly, their corresponding dihydroxy fatty acids take part in the reticulation of this biopolymer [3]. Until lately, however, the enzymes responsible for the epoxidation of free unsaturated fatty acids and their conversion into *vic*-diols were unknown. We have recently reported that peroxygenase, a membrane-bound hydroperoxide-dependent oxygenase purified from soybean (*Glycine max*) seedlings, actively catalysed the epoxidation of *cis*-double bonds of unsaturated fatty acids [4]. Despite their physiological importance, epoxide hydrolases (EC 3.3.2.3) have been surprisingly poorly investigated in higher plants. This contrasts with the wealth of information available for this class of enzymes in mammalian systems (for reviews see, e.g., [5,6]). One of the rare reports on epoxide hydrolysis in plants was by Croteau & Kolattukudy [7]; it concerns the hydration of 9,10-epoxy-18-hydroxystearic acid to *threo*-9,10,18-trihydroxystearic acid catalysed by a particulate fraction from apple (*Malus* sp.) skin [7]. We describe here the occurrence of both membrane-bound and soluble fatty acid epoxide hydrolases in soybean seedlings and the purification to apparent homogeneity of the soluble form.

EXPERIMENTAL

Materials

Elaidic acid, *threo*-9,10-dihydroxystearate, *erythro*-9,10-dihydroxystearate and octyl-Sepharose CL-4B were purchased from Sigma. Oleic acid was from Aldrich. [1-^{14}C]Oleic acid (45 Ci/mol) was obtained from CEA (Saclay, France). [1-^{14}C]18-Hydroxy-*cis*-9-octadecenoate was kindly given by Dr. J. L. Boucher and Dr. D. Mansuy (Université René Descartes, Paris, France). DEAE-Trisacryl M and Ultrogel AcA 44 were from IBF (Villeneuve-la-Garenne, France). [1-^{14}C]*cis*-9,10-Epoxystearic acid, [1-^{14}C]*cis*-9,10-epoxy-18-hydroxystearic acid and unlabelled epoxides were prepared according to standard procedures by oxidation of the fatty acids with monoperoxyphthalic acid (Aldrich) and purified by t.l.c. on silica-gel plates (60 F_{254}; Merck) using diethyl ether/n-hexane/formic acid (70:30:1, by vol.) as solvent system (Solvent A).

Assay for epoxide hydrolase activity

The hydrolase activity was routinely measured by monitoring, by radio-t.l.c., the conversion of a radiolabelled epoxide into its corresponding diol. The enzyme fraction was incubated in 0.1 M-potassium phosphate buffer, pH 7.4, with 2.5 μM-[1-^{14}C]*cis*-9,10-epoxystearic acid (2.5×10^4 d.p.m. in 1 μl of ethanol) at 25 °C, in a final volume of 50 μl. The reaction was stopped by addition of 50 μl of acetonitrile. In the case of soluble epoxide hydrolase the totality of the reaction mixture was applied on a t.l.c. plate and developed in Solvent A. The R_F for the epoxide and its corresponding diol were about 0.65 and 0.25 respectively. For the microsomal hydrolase, after the reaction was stopped by acetonitrile, saturating amounts of NaCl were added to the reaction mixture, which was then extracted with 3×2 vol. of ethyl acetate. The organic extract was then chromatographed as described above. Under these experimental conditions more than 90% of the labelled compounds were recovered.

Identification of 9,10-dihydroxyoctadecanoate and 9,10,18-trihydroxyoctadecanoate was confirmed by g.l.c.–m.s.. The hydroxy fatty acids were first methylated by etheral diazomethane and then treated with small volumes (50–100 μl) of *NO*-bis(trimethylsilyl)trifluoroacetamide (Pierce) for 45 min at room temperature. The mass spectra obtained were in agreement with the literature [8,9], in particular they present prominent fragment ions at m/z 259 and 215 (for the dihydroxy derivative) and 259 and 303 (for the trihydroxy derivative), which are characteristic for the scission of the molecules between the *vic*-trimethylsilyl ethers.

Kinetics

Initial rates were determined under conditions where rates were proportional to enzyme concentrations (up to 1 μg of protein was tested with the purified soluble enzyme) and the reaction progress was linearly dependent upon time (less than 15% of substrate transformation). Pseudo-first-order rate constants $V_{max.}/K_m$ (specificity constant) were also determined from progress curves by using an initial substrate concentration ($[S]_0$) of 1 μM (i.e. $[S]_0 \ll K_m$). Under these conditions $V_{max.}/K_m$ is obtained from the slope of a plot of $\ln([S]_0/[S])$ against $(V_{max.}/K_m)\,t$ [10].

Enzyme purification

Microsomes and supernatants obtained at 100000 *g* were prepared from soybean seedlings as described in [11]. All subsequent steps were performed at 4 °C. The membrane preparations were carefully washed by resuspension in 100 ml of 0.1 M-sodium pyrophosphate buffer, pH 7.4, followed by the same volume of hypo-osmotic medium (0.01 M-potassium phosphate, pH 7.4). The final high-speed supernatant was subjected to fractionation with solid $(NH_4)_2SO_4$. The supernatant of a 40%-satn. step was adjusted to 60% saturation in $(NH_4)_2SO_4$. After stirring for 30 min at 0 °C, the precipitate was collected by centrifugation.

Hydrophobic chromatography. The pelleted proteins were redissolved in a minimal volume of 0.01 M-potassium phosphate buffer, pH 7.4, containing 1.56 M-$(NH_4)_2SO_4$ (buffer A), and immediately applied to a 1.8 cm × 3.5 cm octyl-Sepharose column equilibrated with buffer A. The eluate was recycled over the column, overnight, at a flow rate of 0.5 ml/min. Unbound material was then removed by elution with buffer A. Proteins were eluted with 80 ml of 0.01 M-phosphate buffer, pH 7.4, containing 0.78 M-$(NH_4)_2SO_4$, followed by the same buffer devoid of $(NH_4)_2SO_4$.

DEAE-Trisacryl step. The pooled active fractions, after overnight dialysis against 1.2 litres of 0.01 M-Tris/HCl, pH 8 (buffer B), were applied (0.4 ml/min) to a 1.8 cm × 5.5 cm DEAE-Trisacryl M column equilibrated with buffer B. Unbound material was removed by elution with the same buffer (100 ml). Then a step (32 ml) of buffer B containing 0.1 M-NaCl was applied, followed by a step (48 ml) of buffer B containing 0.3 M-NaCl, which eluted the enzyme.

Gel-filtration step. The active fractions (15 ml) were pooled in a dialysis bag and concentrated to a volume of about 1 ml by using a bed of carboxymethylcellulose. The epoxide hydrolase was finally submitted to gel filtration on a 1.8 cm × 60 cm Ultrogel AcA 44 column equilibrated and eluted with 0.01 M-potassium phosphate buffer, pH 7.4, at a flow rate of 0.25 ml/min. Fractions (1 ml each) were collected and the pooled peak of activity was used immediatedly or kept at −80 °C. The same column, calibrated with protein markers (cytochrome *c*, soybean trypsin inhibitor, ovalbumin, BSA monomer and dimer), was used to determine the apparent molecular mass of the purified native epoxide hydrolase. No change in M_r was observed when the column was eluted in the presence of higher salt concentrations, such as 0.15 M-KCl.

Protein concentrations were measured [12] using BSA (Sigma) as standard. The pI was determined by isoelectric focusing with a PhastSystem (Pharmacia). SDS/PAGE was performed by the method of Laemmli [13]. Gels were silver-stained as described in [14].

Analytical procedures

G.l.c. analyses of the fatty acid epoxide methyl esters and their diol derivatives were performed on a Carlo–Erba (Fractovap 4160) chromatograph apparatus equipped with a Spectra Physics (SP 4270) integrator. The sample was injected directly into the DB-1-coated fused silica capillary column (30 m; 0.25 mm internal diam.; J.W. Scientific) at 60 °C, and the analysis was performed (H_2, 2 ml/min) with the following temperature program: 30 °C/min to 150 °C, followed by 5 °C/min to 280 °C. Under these conditions the retention times of the methyl esters of *cis*- and *trans*-9,10-epoxystearate were respectively 16.03 and 15.8 min, whereas those of the methyl esters of the silylated derivatives of *threo*- and *erythro*-9,10-dihydroxystearate were 19.28 and 19.56 min. G.l.c.–m.s. was performed on a LKB 9000S apparatus with an ionizing energy of 70 eV. The separations were carried out on a DB-5 fused capillary column (30 m; J.W. Scientific) operated between 100 and 300 °C at the rate of 4 °C/min. Radioactivity was read on t.l.c. plates by a Berthold t.l.c. linear analyser (LB 283) and the peaks integrated by a data-acquisition system (LB512).

RESULTS

Occurrence of epoxide hydrolase activities in soybean seedlings

Subcellular fractions of soybean seedlings are capable of readily transforming *cis*-9,10-epoxystearate into 9,10-dihydroxystearate. The structure of the latter compound, which co-migrates on t.l.c. with an authentic standard, has been confirmed by g.l.c.–m.s. (see the Experimental section). The majority of epoxide hydrolase activity was observed in the 100000 *g* supernatant (Table 1). Careful and repeated washings of the microsomal fraction with a pyrophosphate buffer and a hypo-osmotic medium, which totally eliminates soluble enzymes such as peroxidases adsorbed on (or entrapped within) these membranes [11], resulted in a finite epoxide hydrolase specific activity (Table 1). The microsomal activity, which cannot be dissociated from the membranes by further washing (results not shown), accounts for about 1/300th of total cytosolic activity and could therefore represent a membrane-bound form of a hydrolase.

Purification of the soluble epoxide hydrolase

The fatty acid epoxide hydrolase was purified to apparent homogeneity according to the scheme outlined in Table 2. $(NH_4)_2SO_4$ fractionation of the soybean seedling cytosol resulted in the precipitation of the epoxide hydrolase in the 40–60%-satn. fraction. A hydrophobic-interaction chromatographic run on octyl-Sepharose proved an essential step in this purification. The enzyme was adsorbed on the gel in a buffer 40% saturated in $(NH_4)_2SO_4$ and the proteins were fractionated by step elutions [40, 20 and 0% satd. $(NH_4)_2SO_4$]. The major part of the hydrolase activity was eluted as a sharp peak in the latter step (Fig. 1). Further chromatography on an anion-exchange column was performed with a stepwise elution technique of increasing NaCl concentrations which proved superior to a gradient. An apparently homogeneous form of the enzyme was obtained after a gel-filtration step on Ultrogel AcA 44, with a purification factor of about 900. The molecular mass of the native epoxide hydrolase

Table 1. Distribution of fatty acid epoxide hydrolase activity in soybean subcellular fractions

The enzyme was purified starting from 30 g dry weight of soybean seeds. The activity was assayed with 2.5 μM-9,10-epoxystearate as substrate.

Fraction	Total activity (μmol/min)	Total activity (%)	Specific activity (nmol/min per mg of protein)
Crude extract	12.53	100	3.4
Centrifugation at 100000 *g*			
Supernatant	10.8	86	3.4
Pellet	1.6	13	3.3
Centrifugation at 100000 *g*			
Supernatant	9.4	75	2.2
Microsomes	0.4	3	0.58
Microsomal washing			
First	0.05	0.40	0.92
Second	0.03	0.24	0.52

Table 2. Representative purification of soluble fatty acid epoxide hydrolase from soybean seedlings

The enzyme was purified starting from 30 g dry weight of soybean seeds. The activity was assayed with 2.5 μM-9,10-epoxystearate as substrate.

Purification stage	Protein (mg)	Total activity (μmol/min)	Total activity (%)	Specific activity (nmol/min per mg)	Purification factor (fold)
Supernatant	2170	4.50	100	2.1	1
$(NH_4)_2SO_4$ (40–60% satn.)	214	1.35	30	6.3	3
Octyl-Sepharose	14.7	1.24	27	84.5	40
DEAE-Trisacryl	1.6	0.97	22	596	284
Ultrogel AcA 44	0.24	0.44	10	1870	900

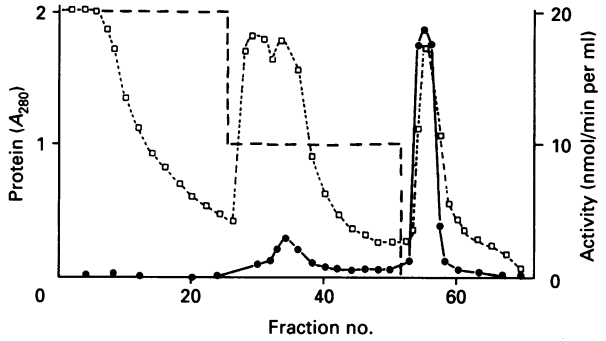

Fig. 1. Hydrophobic chromatography of soluble epoxide hydrolase

The enzyme was purified on an octyl-Sepharose column as described in the Experimental section. The column was eluted with 0.01 M-potassium phosphate buffer, pH 7.4, containing 1.56 M-$(NH_4)_2SO_4$ (fractions 1–25) or 0.78 M-$(NH_4)_2SO_4$ (fractions 26–52) (----); the enzyme was eluted with the same buffer, but devoid of $(NH_4)_2SO_4$. Fractions (3 ml) were assayed for hydrolase activity (●) and protein (□).

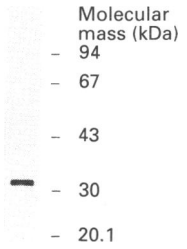

Fig. 2. Analysis of purified epoxide hydrolase by SDS/PAGE

The enzyme ($\approx$ 1 μg) was analysed under denaturing conditions on a 10%-(w/v)-polyacrylamide gel and its molecular mass was estimated relative to standard proteins.

determined by gel filtration was 64$\pm$2 kDa (n = 3). The native enzyme revealed a single band on isoelectric focusing with a pI of 5.4, and SDS/PAGE analysis gave one band with a molecular mass of 32$\pm$1 kDa (n = 3) (Fig. 2). This suggests that the soluble soybean fatty acid epoxide hydrolase is a dimer.

Characterization of the epoxide hydrolase

By using *cis*-9,10-epoxystearate as a variable substrate, K_m(app.) values of 26$\pm$5 and 45$\pm$7 μM were obtained respectively for the microsomal and the soluble hydrolases (Fig. 3). The respective $V_{max.}$ values were 6.1$\pm$0.7 nmol/min per mg of protein

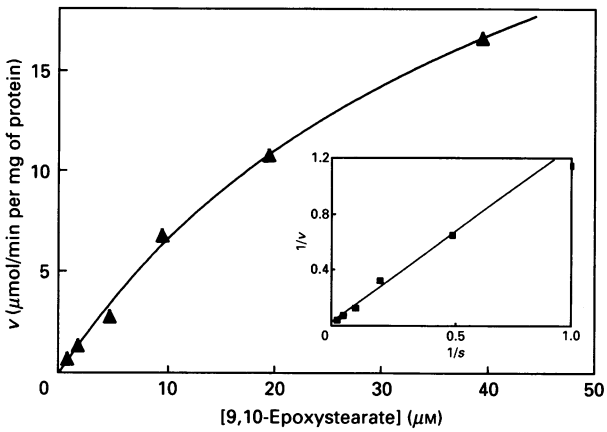

Fig. 3. Substrate-concentration-dependence for the purified soluble epoxide hydrolase activity

The assays were performed as described in the Experimental section at the indicated concentrations. The kinetic parameters were calculated by fitting the Michaelis–Menten equation using a non-linear-regression-analysis program (the inset shows a Lineweaver–Burk plot).

and 35$\pm$4 μmol/min per mg of protein respectively. One should be aware that such determinations yield only apparent values. The difficulties encountered with hydrophobic substrates and also with membrane-bound enzymes, namely anisotropic solutions and K_m(app.) dependency on the amount of microsomal membranes in the assay mixture, have been documented previously [15]. Both hydrolases present an optimal pH near 7.5 (see Fig. 4 for the soluble enzyme).

Substrate specificity. Purified soybean epoxide hydrolase hydrates *cis*-9,10-epoxystearate exclusively into *threo*-9,10-dihydroxystearate. The configuration of the reaction product was established by g.l.c. analysis by comparing its retention time with that of an authetic sample after derivatization (see the Experimental section). In contrast, *trans*-9,10-epoxystearate was a poorer substrate of this enzyme. This could be ascribed in part to the low affinity of this compound for the active site of the hydrolase; for example, the hydration of 25 μM-[1-^{14}C]*cis*-9,10-epoxystearate (about K_m/2) was inhibited by only 20% with 1 mM-*trans*-9,10-epoxystearate. At a high concentration (35 mM) the *trans*-epoxide was, however, found (using the same g.l.c. technique) to be readily converted into *erythro*-9,10-dihydroxystearate. The rate observed was about 45% of the maximal rate for *cis*-9,10-epoxystearate.

In order to compare the soluble soybean epoxide hydrolase with the particulate enzyme previously described by Croteau &

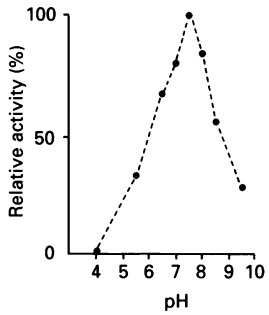

Fig. 4. pH-dependence for the purified soluble epoxide hydrolase activity

The enzyme was assayed with 9,10-epoxystearate in the presence of 0.1 M-citrate (pH 4.0–5.5), acetate (5.5–6.5), phosphate (6.5–8.0) and glycine (8.0–9.5) buffers (sodium salts were used in each case).

Kolattukudy [7], we assessed its specificity towards *cis*-9,10-epoxystearate and *cis*-9,10-epoxy-18-hydroxystearate. Since the specificity of an enzyme for two competing substrates is controlled by the ratio of their respective $V_{max.}/K_m$ values [16], we determined these rate constants from progress curves at low concentrations (1 μM) of the two substrates (see the Experimental section). A ratio of about 23 ± 2 ($n = 3$) was found is favour of *cis*-9,10-epoxystearate indicating, in sharp contrast with the particulate epoxide hydrolase [7], a strong preference of the soybean hydrolase for this fatty acid epoxide.

Inhibitors. Styrene oxide and *trans*-stilbene oxide (up to 0.4 mM), which are good substrates for respectively the microsomal and soluble epoxide hydrolases of mammalian origin [5,6], were not found to compete for the hydration of 9,10-epoxystearate (at $K_m/2$) catalysed by either the soybean soluble or membrane-bound enzyme. The plant enzyme was inhibited by neither of the thiol reagents *N*-ethylmaleimide (10 mM) and *p*-mercuribenzoate (1 mM). However *m*-chloroperbenzoic acid was found to be a very powerful inactivator of this enzyme (e.g. at 10 μM this molecule totally inactivated the enzyme within 5 min).

DISCUSSION

The common occurrence in seed oils and plant extracts of *vic*-diols derived from fatty acids and having the *threo*-configuration suggested the presence of epoxide hydrolases capable of hydrating epoxidized fatty acids having a *cis*-configuration. We have demonstrated the presence of such an activity in soybean seedlings and purified to apparent homogeneity a soluble form of an epoxide hydrolase which efficiently converts *cis*-9,10-epoxystearate into *threo*-9,10-dihydroxystearate. The stereospecificity of this oxirane ring opening is in accordance with the known *trans*-hydration catalysed by mammalian epoxide hydrolases [5,6]. Importantly, the specific activity of the purified soybean epoxide hydrolase (about 35 μmol/min per mg of protein) is among the highest yet reported for that class of enzymes [6]. In contrast with the mammalian epoxide hydrolases, for which few endogenous substrates are known (e.g. epoxides derived from arachidonic acid or 5,6α-epoxycholesterol [6]), the soybean epoxy fatty acid hydrolase described here is rather specific and active on metabolites which are of physiological relevance. Comparatively with the cytosolic epoxide hydrolase, which in animals is active (but not exclusively) on a wide variety of aliphatic epoxides, including 9,10-epoxystearate [17,18], the soybean enzyme has a similar pI, is also a dimer, but has a lower molecular mass [5,6]. The properties of the soybean epoxide hydrolases, i.e. subcellular localization, substrate specificity and optimal pH, clearly distinguish this enzyme from the epoxide hydrolase described by Croteau & Kolattukudy in particulate fractions of apples [7].

In addition to the soluble form, a minor fraction of epoxide hydrolase of similar specificity was found firmly associated with the microsomes (specific activity 6 nmol/min per mg of protein). This location could ensure a ready access to fatty acid epoxides generated by membrane-bound oxygenases such as peroxygenase. The purification and structural properties of this membrane form of fatty acid epoxide hydrolase need to be further investigated. It is noteworthy in this context that, in rat liver, microsomal and soluble epoxide hydrolases of same substrate specificity were also reported [19].

In conclusion, this new plant epoxide hydrolase, which is active on endogenous substrates, should provide a better insight into the metabolism of fatty acid derivatives involved in defence mechanisms and cutin biosynthesis.

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REFERENCES

1. Smith, C. R., Jr. (1970) Prog. Chem. Fats Lipids **11**, 137–177
2. Kato, T., Yamaguchi, Y., Uyehara, T., Yokoyama, T., Namai, T. & Yamanaka, S. (1983) Naturwissenschaften **70**, 200–201
3. Kolattukudy, P. E. (1981) Annu. Rev. Plant Physiol. **32**, 539–567
4. Blée, E. & Schuber, F. (1990) J. Biol. Chem. **265**, 12887–12894
5. Wixtrom, R. N. & Hammock, B. D. (1981) Biochem. Pharmacol. Toxicol. **1**, 1–93
6. Meijer, J. & DePierre, J. W. (1988) Chem.-Biol. Interact. **64**, 207–249
7. Croteau, R. & Kolattukudy, P. E. (1975) Arch. Biochem. Biophys. **170**, 61–72
8. Holloway P. J. (1982) in The Plant Cuticle (Cutler, D., Alvin, K. & Price, C., eds.), pp. 45–85, Academic Press, London and New York
9. Yruela, A. B. & Grimalt, J. O. (1990) J. Chromatogr. Sci. **28**, 421–427
10. Orsi, B. A. & Tipton, K. F. (1979) Methods Enzymol. **63**, 159–183
11. Blée, E. & Schuber, F. (1989) Biochemistry **28**, 4962–4967
12. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
13. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
14. Morrisey, J. H. (1981) Anal. Biochem. **117**, 307–310
15. Lu, A. Y. H., Jerina, D. M. & Levin, W. (1977) J. Biol. Chem. **252**, 3715–3723
16. Fersht, A. (1977) Enzyme Structure and Mechanism, chapter 3, Freeman, San Francisco
17. Gill, S. & Hammock, B. D. (1979) Biochem. Biophys. Res. Commun. **89**, 965–971
18. Watabe, T., Komatsu, T., Isobe, M. & Tsubaki, A. (1983) Chem.-Biol. Interact. **44**, 143–154
19. Guenthner, T. M. & Oesch, F. (1983) J. Biol. Chem. **258**, 15054–15061

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