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ENANTIOSELECTIVITY OF THE HYDROLYSIS OF LINOLEIC ACID MONOEPOXIDES CATALYZED BY SOYBEAN FATTY ACID EPOXIDE HYDROLASE

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Soybean epoxide hydrolase efficiently catalyzes the hydration of the two positional isomers of linoleic acid monoepoxides into their corresponding *vic*-diols. Kinetic analysis of the progress curves, obtained at low substrate concentrations (*i.e.* $[S_o] << Km$), and analysis of the residual substrates by chiral-phase HPLC, indicate that the hydrolase is highly enantioselective, i.e. *cis*-9*R*,10*S*-epoxy-12(Z)-octadecenoic and *cis*-12*R*,13*S*-epoxy-9(Z)-octadecenoic acids are preferentially hydrolyzed (the enantioselectivity ratios are 15 and 28, respectively). Importantly, these two enantiomers are the one formed preponderantly by epoxidation of linoleic acid by peroxygenase, a hydroperoxide-dependent oxidase we have previously described in soybean (Blée, E., and Schuber, F., *Biochem. Biophys. Res. Commun.* (1990) **173**, 1354-1360).

We have recently shown that peroxygenase, a strictly hydroperoxide-dependent membrane-associated oxidase occurring in higher plants, was able to efficiently catalyze the epoxidation of mono- and polyunsaturated fatty acids [1]. Thus, linoleic acid was transformed into 9,10-epoxy-12(Z)-octadecenoic acid (coronaric acid) and 12,13-epoxy-9(Z)-octadecenoic acid (vernolic acid). Importantly, such compounds have been found to be involved in the host's defense against fungal and bacterial pathogens. For example, they are inhibitors of the spore germination and germ tube growth of rice blast disease (*Pyricularia oryzae*) [2-4]. 9,10-Epoxy-12-octadecenoate is also found in mammals, synthesized e.g. by neutrophils [5]; this molecule, known as leukotoxin, was recently proposed as a burn toxin [6]. Linoleate epoxides seem also toxic for plant tissues and it has been suggested that their accumulation could be controlled by their hydrolysis [7]. Interestingly, their corresponding diol derivatives still keep antifungal properties [2]. Recently we have described, in soybean, the occurrence of epoxide hydrolases specific for fatty acid epoxides [8] and studied the molecular mechanism of 9,10-epoxystearate hydration catalyzed by the purified soluble form of this hydrolase [9].

In this paper, we have examined the enantioselectivity of the hydrolysis of 9,10-epoxy-12(Z)-octadecenoic and 12,13-epoxy-9(Z)-octadecenoic acids catalyzed by this new epoxide hydrolase. The results obtained indicate that the enzyme hydrates preferentially the 9*R*,10*S* and 12*R*,13*S* enantiomers, which are precisely the major metabolites formed by epoxidation of linoleic acid by soybean peroxygenase [13].

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EXPERIMENTAL PROCEDURES

Materials - [1-^{14}C]Linoleic acid (56 Ci/mol) was purchased from CEN-Saclay (Gif sur Yvette, France); linoleic acid was from Sigma (France).

Enzyme preparations - Microsomes and high-speed (10^5x*g*) centrifugation supernatants were prepared from soybean (*Glycine max*) seedlings as published before [8,10]. Peroxygenase was solubilized with emulphogene BC 720 from carefully washed microsomes and partially purified [1,10] by ion-exchange chromatography (CM-Sepharose). Epoxide hydrolase was purified from the supernatant as previously described [8].

Preparation of linoleic acid monoepoxides - Racemic ^{14}C-labeled and unlabeled *cis*-monoepoxides were prepared according to standard procedures by oxidation of linoleic acid with monoperoxyphtalic acid (Aldrich). 9,10-Epoxy-12-octadecenoic and 12,13-epoxy-9-octadecenoic acids were separated by silica gel TLC (60 F_{254} Merck) using a *n*-hexane/diethyl ether/formic acid (50:50:1) solvent system (system A): Rf= 0.37 and Rf= 0.43 respectively. Enzymatic [1-^{14}C]linoleic acid monoepoxides were obtained by incubating [1-^{14}C]linoleic acid (90μM, 7.8x10^6dpm) in 10mM sodium acetate buffer (pH 5.5) and 0.1 % emulphogene (final volume 1ml) in the presence of cumene hydroperoxide (1 mM) and partially purified peroxygenase (40μg protein) or microsomal preparations (0.5 mg protein). After work-up [1], the two positional isomers of linoleic acid monoepoxides were separated by TLC using system A.

Identification of the hydrolysis reaction products - Each positional isomer of linoleate monoepoxides (30μg) was hydrolyzed by purified soybean epoxide hydrolase (50μg) in a final volume of 0.1ml of 0.1M KH_2PO_4 (pH 7.4). After 15 min at 26° C, the reaction was stopped by 4N HCl (pH 3-4) and the mixture extracted with diethyl ether. The reaction products were purified by TLC as above. After treatment with ethereal diazomethane and derivatization with N,O-bis(trimetylsilyl)trifluoro-acetamide (Pierce), identity of 9,10-dihydroxy-12-octadecenoate and 12,13-dihydroxy-9-octadecenoate were confirmed by GC/MS. The mass spectra obtained were in agreement with the literature [11,12], in particular they present characteristic fragment ions at *m/z* 299, 275 and 173 (for 12,13-dihydroxy-9-octadecenoate) and 361, 259 and 213 (for 9,10-dihydroxy-12-octadecenoate) which result from the scission of the molecules between the *vic*-trimethylsilyl ethers.

Kinetics of epoxide hydrolase - Progress curves of the hydrolysis of [1-^{14}C]*cis*-9,10-epoxy-12(*Z*)-octadecenoate and [1-^{14}C]*cis*-12,13-epoxy-9(*Z*)-octadecenoate were determined under conditions where $[S_o] << K_m$. For a typical assay the enzyme was pre-equilibrated for 1 min, at 26° C, in 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 600 μl. The reaction was initiated by addition of 2 μM ^{14}C-labeled substrate. At given times, 50 μl aliquots were withdrawn and the reaction stopped with 50 μl of acetonitrile. The mixture was then applied to a silica gel TLC plate developed in solvent system A. Radioactivity corresponding to the diols (Rf=0.13 and 0.15 for respectively 9,10-and 12,13-dihydroxyoctadecenoates) and the residual monoepoxides were then determined. Under these assay conditions reaction rates were linear with respect to protein concentrations. In the absence of enzyme activity (boiled enzyme), no transformation of the epoxides were detectable.

When using these substrate concentrations, progress curves can be analyzed [9,14] according to the Equation 1: $P(\%) = A(1 - \exp[k_A t]) + B(1 - \exp[k_B t])$, where *P* represents reaction progress, *i.e.* formation (in percentage) of the diol products as a function of time (*t*), and k_A and k_B represent the pseudo-first-order rate constants for the hydrolysis of, respectively, the fast and the slow enantiomer. A and B are, in percentage, the proportions of the two enantiomers; *e.g.* for a racemic substrate, A and B = 50. The k_A and k_B values were calculated by fitting, with a nonlinear regression program, the experimental data to Eq. 1. The specificity constants $(V/K_m)_A$ and $(V/K_m)_B$ were obtained by the relation: $V/K_m = k/[E]$ where [E] is the enzyme concentration.

Analytical procedures - Radioactivity was read on TLC plates by a Berthold TLC linear analyzer LB 283, and the peaks were integrated by a data acquisition system LB 512. GC/MS was performed on a LKB 9000S apparatus with ionizing energy of 20 eV. The separations were carried out on a DB-5 fused silica capillary column (30 m, J.W. Scientific) operated between 100 and 250° C at the rate of 4° C/min. Chiral-phase HPLC was performed at room temperature, on a Shimadzu instrument, under isocratic elution con-

ditions. Each ^{14}C-labeled monoepoxide of linoleic acid was first methylated in presence of ethereal diazomethane, then their resolution was achieved on a Chiralcel OB column (4.6x 250 mm; Baker Chemical Co.) with a solvent mixture of 0.04 % isopropanol in *n*-hexane at 0.7 ml/min. In this system, elution times for methyl 9*S*,10*R* or 9*R*,10*S*-epoxy-12-octadecenoate and methyl 12*S*,13*R* or 12*R*,13*S*-epoxy-9-octadecenoate were respectively 62, 77, 83 and 98 min. Radiochromatograms and peak area integrations were obtained using Flo-one ß-detector with the program Boreal (Flotec, France).

RESULTS AND DISCUSSION

In analogy with the transformation of *cis*-9,10-epoxystearate [9,13], soybean fatty acid epoxide hydrolase was found to efficiently catalyze the hydration of *cis*-9,10-epoxy-12 (*Z*)-octadecenoic and *cis*-12,13-epoxy-9(*Z*)-octadecenoic acids. The identity of the reaction products, *i.e.* the corresponding *vic*-diols, was established by GC/MS (see "Experimental Procedures"). In order to study the enantioselectivity of the hydrolase, *i.e.* its ability to discriminate between the two enantiomers of each positional isomer of linoleic acid monoepoxides, we have determined for each enantiomer its specificity constant V/K_m. Indeed, as analyzed by Fehrst [15], the ability of an enzyme to discriminate kinetically between two substrates A and B competing for a same active site is given by Equation 2: $v_A/v_B = (V/K_m)_A[A]/(V/K_m)_B[B]$. Accordingly, for each linoleic acid monoepoxide we have determined the enantioselectivity ratio; i.e. $(V/K_m)_A/(V/K_m)_B$ where A and B are respectively the fast and slow reacting enantiomer. The individual V/Km ratios were obtained from progress curves as described under "Experimental Procedures".

Hydrolysis of cis-*9,10-epoxy-12(Z)-octadecenoate* - We have followed, by TLC-radiochromatography, the hydrolysis of racemic [1-^{14}C]-9,10-epoxy-12-octadecenoic acid by soybean epoxide hydrolase. Fig. 1 shows that biphasic kinetics were observed, with a

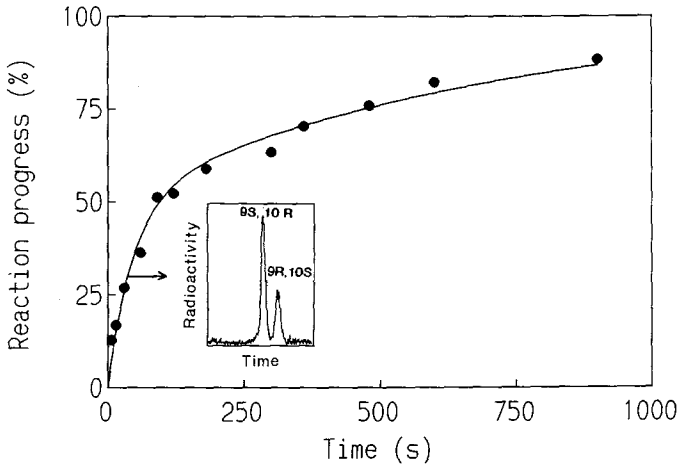

Fig. 1. **Progress curve of the hydrolysis of racemic [1-^{14}C]*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid catalyzed by soybean epoxide hydrolase.** Substrate (2 μM) was incubated at 26° C in the presence of enzyme (4.6 μg of protein) in 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 600 μl. Aliquots were taken at given times and analyzed by TLC as indicated under "Experimental Procedures". The solid line represents the theoretical curve obtained by fitting, with a nonlinear regression program, the data to Equation 1. *Inset*: Chiral phase HPLC of the unreacted epoxide after 30% reaction progress.

Table I

Summary of the kinetic data for the hydrolysis of substrates by soybean fatty acid epoxide hydrolase

Substrate	$(V/K_m)_A$	$(V/K_m)_B$	Enantioselectivity ratio
	min⁻¹/mg protein/ml		
cis-9,10-Epoxystearic acid	695	44.2	15.7 (9*R*,10*S*/9*S*,10*R*)
cis-9,10-Epoxy-12(*Z*)-octadecenoic acid	318.7	21.8	14.6 (9*R*,10*S*/9*S*,10*R*)
cis-12,13-Epoxy-9(*Z*)-octadecenoic acid	1418	50.6	28.0 (12*R*,13*S*/12*S*,13*R*)

The specificity constants (V/K_m) for hydrolysis (at pH 7.4 and 26°C)of the racemic substrates, under pseudo-first-order kinetic conditions, were calculated from the progress curves as described under Materials and Methods. Assignment of the more reactive enantiomer was accomplished by chiral phase HPLC of the unreacted substrate (see text). The results are the average of two independent determinations.

marked transition around 50% of reaction progress. A similar behavior was found during hydration of racemic 9,10-epoxystearate [9], it is characteristic for an enantioselective enzyme which hydrolyzes preferentially one epoxide enantiomer during the first half of the reaction. For this linoleic acid monoepoxide an enantioselectivity ratio of 14.6 was calculated (Table I), which is very similar to the one, under identical conditions, for 9,10-epoxystearic acid (*i.e.* 15.7; Table I). Thus, the presence of an extra 12,13(*Z*)-double bond in the 9,10-epoxide molecule has little influence on the enantioselectivity of the epoxide hydrolase. In terms of specificity however, 9,10-epoxy-12-octadecenoate was somewhat a poorer substrate of the hydrolase; *i.e.*, the $(V/K_m)_A$ ratio (Table I) was about 2.2, in favor of 9,10-epoxystearate.

The identity of the fast reacting enantiomer was established by analyzing, by chiral phase HPLC on a Chiralcel OB-type column, the absolute configuration of the unreac-

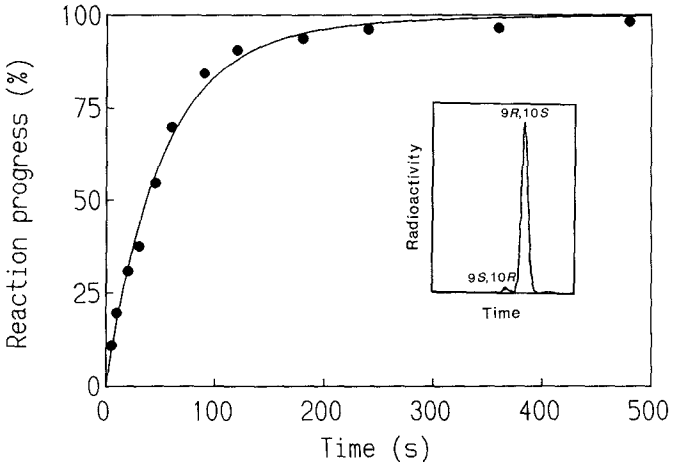

Fig. 2. **Progress curve of the hydrolysis of [1-^{14}C]*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid prepared by peroxygenase-catalyzed epoxidation of linoleic acid.** The experimental conditions were identical to those of Fig. 1. *Inset*: Analysis by chiral phase HPLC of the substrate.

ted 9,10-epoxy-12-octadecenoate. Racemic 9,10-monoepoxide of linoleic acid could be resolved into two peaks of equal importance on this type of column (not shown). The configurational assignment of the peaks was established knowing the enantioselective epoxidation of linoleic acid by soybean peroxygenase, *i.e.* the partially purified enzyme produces the 9*R*,10*S*-epoxy-12(*Z*)-octadecenoate with over 80% ee [13] and, as shown in Fig. 2 (Inset), this enantiomer elutes last. Fig. 1 (Inset) indicates that this enantiomer is precisely the one which is hydrolyzed first by the epoxide hydrolase. This was confirmed by studying the hydrolysis of the 9,10-monoepoxide prepared with the peroxygenase. As shown in Fig. 2 the progress curve is quasi-monoexponential, *i.e.* no change in slope was observed at 50% of reaction progress.

Hydrolysis of cis-*12,13-epoxy-9(Z)-octadecenoate* - The resolution by chiral phase HPLC of the racemic and peroxygenase-synthesized 12,13-epoxy-9-octadecenoic acid are shown in Fig.3A and 3B, respectively. Since we have previously determined that partially purified soybean peroxygenase catalyzes preferentially the formation of the 12*R*,13*S*-enantiomer (a 65:35 ratio in favor of this epoxide was observed [13]), it follows that the enantiomer possessing the *R,S* configuration elutes again last. The progress

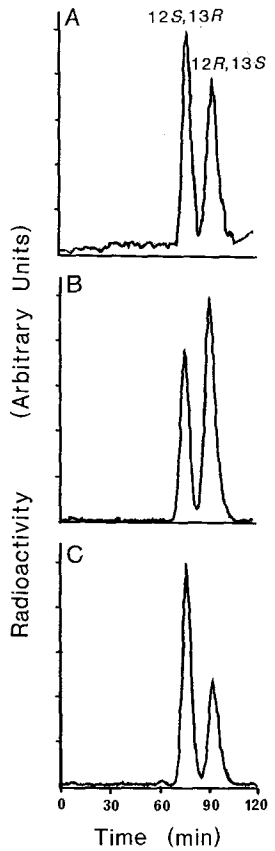

Fig. 3. **Chiral phase HPLC of [1-^{14}C]*cis*-12,13-epoxy-9(*Z*)-octadecenoic acids.** The radiochromatograms represent the racemic epoxide (A), and the epoxide obtained by partially purified peroxygenase (B) or microsomes (C) catalyzed monoepoxidation of linoleic acid (see "Experimental Procedures").

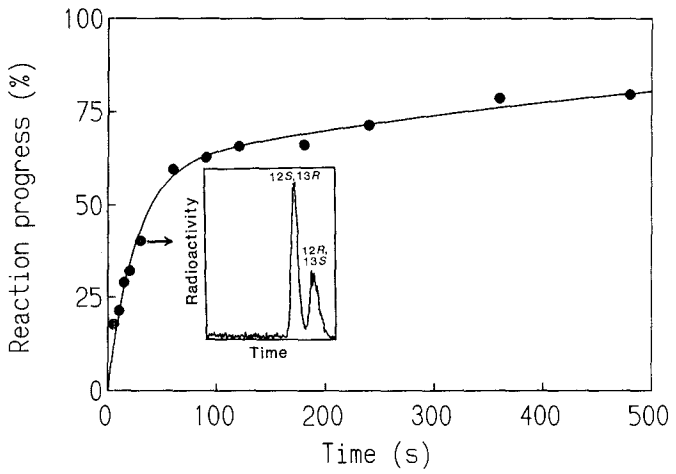

Fig. 4. **Progress curve of the hydrolysis of [1-^{14}C]*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid prepared by peroxygenase-catalyzed epoxidation of linoleic acid.** The experimental conditions were the same as those in Fig.1(concentration of enzyme was 1.5 μg). *Inset*: Chiral phase HPLC of the unreacted epoxide after 40% reaction progress.

curve of the hydrolysis of the enzymatic 12,13-epoxy-9-octadecenoic acid by soybean epoxide hydrolase in given in Fig. 4. A strong enantioselectivity was again observed (ratio = 28, Table I) in favor of one of the enantiomers. The fast reacting enantiomer has the 12*R*,13*S* configuration: i) a marked rupture of the progress curve was observed only after about 65% reaction progress (Fig.4) and ii) analysis by chiral phase HPLC of the residual epoxide after 40% conversion into the diol (Fig.4, Inset) indicates a decrease in the height of the peak corresponding to the 12*R*,13*S*-enantiomer. It results that the 12,13-epoxide formed from linoleic acid by the soybean peroxygenase is also the preferred substrate for the epoxide hydrolase. Moreover, in terms of specificity, analysis of the respective $(V/K_m)_A$ values, indicates that, compared to the 9,10-monoepoxide of linoleic acid, the 12,13-regioisomer is the favored substrate of the epoxide hydrolase ($(V/K_m)_A$ ratio = 4.4; Table I). Interestingly, in the epoxidation of linoleic acid the peroxygenase presented a regioselectivity in favor of the 9,10 unsaturation [1].

The present results on the regio- and enantioselectivity of the epoxide hydrolase bear some important consequences on the outcome of regioselectivity and stereochemistry studies when epoxidation reactions are carried out with microsomal peroxygenase. Although epoxide hydrolase is mostly found in the soluble fractions, we have shown that it can contaminate microsomes to variable extents depending on the membrane pellet washing procedures [8]. Moreover a tightly membrane-bound form of the hydrolase was also detected [8], which has an identical enantioselectivity (unpublished results). It follows that using such microsomes, *e.g.* in the epoxidation of linoleic acid, one might *underestimate* both the regioselectivity of the peroxygenase catalyzed epoxidation and the enantiomeric excess of the two monoepoxides. This was borne out experimentally by comparing the stereochemistry of the 12,13-monoepoxides obtained by epoxidation of linoleic acid by partially purified soybean peroxygenase (Fig. 3B) or by microsomes (Fig. 3C). The chiral phase HPLC elution profiles show *opposite* stereochemistries, *i.e.* the peroxygenase yields a 12*R*,13*S*-isomer (30 % ee) whereas microsomes gave a 12*S*,13*R*-

isomer, with a 26% ee in this particular experiment. Such a result can be explained by the favored hydrolysis, by the epoxide hydrolase present in the microsomes, of the 12*R*,13*S*-epoxide enantiomer resulting from the peroxygenase catalyzed monoepoxidation of linoleic acid (diols were detected by TLC; not shown). This result could also clarify the apparent discrepancy between our results on the stereochemistry of 12,13-monoepoxidation of linoleic acid by the peroxygenase [1] and those of Hamberg and Hamberg [16], who used microsomal oxidases.

Conclusion - Soybean fatty acid epoxide hydrolase is a highly enantioselective enzyme. It hydrates preferentially the 9*R*,10*S*- and 12*R*,13*S*-enantiomers of the linoleic acid monoepoxides which are the major reaction products of linoleic acid epoxidation by soybean peroxygenase. Our results suggest that the two enzymes we are studying are excellent candidates to fulfil *in vivo* a fundamental role in the biosynthesis of a class of oxygenated fatty acids known to be involved in plant defense mechanisms. From a practical point of view, the soybean epoxide hydrolase could in principle be exploited to perform kinetic resolutions on racemic linoleic acid monoepoxides. Finally, the resolution developed here, by chiral phase HPLC, of the linoleic acid monoepoxide enantiomers could be extended to the determination of the configuration of epoxides such as leukotoxins.

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