Plant Physiol. (1992) 99, 987–995
0032-0889/92/99/0987/09/$01.00/0

Received for publication October 25, 1991
Accepted January 30, 1992

On the Specificity of a Fatty Acid Epoxygenase in Broad Bean (*Vicia faba* L.)[1]

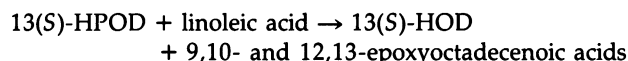
Mats Hamberg* and Per Fahlstadius

Department of Physiological Chemistry, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

ABSTRACT

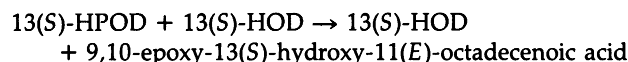
Seeds of broad bean (*Vicia faba* L.) contain a hydroperoxide-dependent fatty acid epoxygenase. Hydrogen peroxide served as an effective oxygen donor in the epoxygenase reaction. Fifteen unsaturated fatty acids were incubated with *V. faba* epoxygenase in the presence of hydrogen peroxide and the epoxy fatty acids produced were identified. Examination of the substrate specificity of the epoxygenase using a series of monounsaturated fatty acids demonstrated that (*Z*)-fatty acids were rapidly epoxidized into the corresponding *cis*-epoxy acids, whereas (*E*)-fatty acids were converted into their *trans*-epoxides at a very slow rate. In the series of (*Z*)-monoenoic acids, the double bond position as well as the chain length influenced the rate of epoxidation. The best substrates were found to be palmitoleic, oleic, and myristoleic acids. Steric analysis showed that most of the epoxy acids produced from monounsaturated fatty acids as well as from linoleic and α-linolenic acids had mainly the (*R*),(*S*) configuration. Exceptions were C_{18} acids having the epoxide group located at C-12/13, in which cases the (*S*),(*R*) enantiomers dominated. 13(*S*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic acid incubated with epoxygenase afforded the epoxy alcohol 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid as the major product. Smaller amounts of the diastereomeric epoxy alcohol 9(*R*),10(*S*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid as well as the α,β-epoxy alcohol 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoic acid were also obtained. The soluble fraction of homogenate of *V. faba* seeds contained an epoxide hydrolase activity that catalyzed the conversion of *cis*-9,10-epoxyoctadecanoic acid into *threo*-9,10-dihydroxyoctadecanoic acid.

A hydroperoxide-dependent fatty acid epoxygenase in broad bean (*Vicia faba* L.) was recently discovered (13). In the presence of added hydroperoxides such as 13(*S*)-HPOD[2] and cumene hydroperoxide, this enzyme catalyzed epoxidation of oleic acid into *cis*-9,10-epoxyoctadecanoic acid. Studies with $^{18}O_2$-labeled 13(*S*)-HPOD showed that the reaction consisted of a direct transfer of hydroperoxide oxygen to the Z double bond of the substrate to provide the *cis*-epoxide function of the product (13). An active ω6-lipoxygenase was present in the membrane preparation of *V. faba* used as source of epoxygenase. Therefore, incubation of linoleic acid with the enzyme preparation resulted in the formation of epoxidized derivatives also in the absence of added hydroperoxide. In the major pathway of linoleic acid metabolism, part of the linoleic acid was converted into 13(*S*)-HPOD by action of the lipoxygenase. The epoxygenase catalyzed subsequent transfer of hydroperoxide oxygen from 13(*S*)-HPOD to another part of linoleic acid, *i.e.* the reaction:



13(*S*)-HPOD + linoleic acid → 13(*S*)-HOD
+ 9,10- and 12,13-epoxyoctadecenoic acids

Another pathway consisted of formation of 9,10-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid. This epoxy alcohol was formed from 13(*S*)-HPOD, partly by intramolecular epoxidation, and partly by an intermolecular epoxidation according to the reaction:



13(*S*)-HPOD + 13(*S*)-HOD → 13(*S*)-HOD
+ 9,10-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid

The present paper reports further studies of the *V. faba* epoxygenase with the emphasis on the substrate specificity and stereospecificity of the enzyme.

MATERIALS AND METHODS

Chemicals

Most of the unlabeled fatty acids were purchased from Nu-Chek-Prep (Elysian, MN). 9(Z)-Dodecenoic acid and 9(*E*)-dodecenoic acid were prepared as recently described (5). A mixture of 9(Z)- and 12(Z)-octadecenoic acids (1:1, w/w) was obtained by semireduction of methyl linoleate with diimide (4) followed by isolation of the monoenoic ester fraction by reversed-phase HPLC (column, 300 × 8 mm Polygosil C_{18} 5μm; solvent system, acetonitrile:water [95:5, v/v]; effluent volume, 16.4–19.2 mL). The material thus obtained was subjected to alkaline hydrolysis and the free acids purified by silicic acid column chromatography. [1-^{14}C] Oleic, [1-^{14}C]linoleic, and [1-^{14}C]α-linolenic acids were obtained from Amersham, UK, and diluted with the corresponding unlabeled acids to make specimens having the specific radioactivities indicated: [1-^{14}C]oleic acid, 3.1 kBq/μmol; [1-^{14}C]linoleic acid, 7.0 kBq/μmol; and [1-^{14}C]α-linolenic acid, 15.9 kBq/μmol. 13(*S*)-HPOD and 13(*S*)-[1-^{14}C]HPOD (chemical and radiochemical purity, >95%; specific radioactivity, 7.0 kBq/μmol) were prepared as previously described (12). 13(*S*)-[1-^{14}C]HOD (purity, >95%; specific radioactivity, 7.0 kBq/μmol) was obtained by reduction of 13(*S*)-HPOD with sodium borohydride followed by purification by silicic acid

[1] This work was supported by the Swedish Medical Research Council (project No. 03X-05170) and by the Swedish Society of Medicine.

[2] Abbreviations: 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9(Z),11(*E*)-octadecadienoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; 13(*S*)-HOD, 13(*S*)-hydroxy-9(Z),11(*E*)-octadecadienoic acid; Me_3Si, trimethylsilyl.

column chromatography. [1-^{14}C]*cis*-9,10-Epoxyoctadecanoic acid (enantiomeric composition, 82% 9(*R*),10(*S*) and 18% 9(*S*),10(*R*)) was prepared by incubation of [1-^{14}C]oleic acid with broad bean (*Vicia faba*) epoxygenase in the presence of cumene hydroperoxide (13) followed by purification by silicic acid column chromatography. (±)-*cis*-10,11-Epoxynonadecanoic acid was prepared by epoxidation of methyl 10(Z)-nonadecenoate (100 mg) with *m*-chloroperbenzoic acid (110 mg) in chloroform (25 mL) at 22°C for 2 h followed by saponification and purification by silicic acid column chromatography. An aliquot was esterified by treatment with diazomethane and analyzed by GC-MS. The C value was 20.55 (reference, methyl (±)-*cis*-9,10-epoxyoctadecanoate, C-19.55), and the mass spectrum showed prominent ions at m/z 326 (M), 295 (M-31; loss of $\cdot OCH_3$), 213 (M-113; loss of $\cdot(CH_2)_7$-CH_3), 201 (HO^+ = CH-$(CH_2)_8$-$COOCH_3$), 185 ([$(CH_2)_9$-$COOCH_3$]$^+$), 169 (201–32; loss of CH_3OH), and 155 (M-171; loss of $\cdot(CH_2)_8$-$COOCH_3$) (*cf.* ref. 7). Methyl (±)-*cis*-9,10-epoxytetradecanoate, methyl (±)-*cis*-9,10-epoxyhexadecanoate, methyl (±)-*cis*-6,7-epoxyoctadecanoate, methyl (±)-*trans*-6,7-epoxyoctadecanoate, methyl (±)-*cis*-9,10-epoxyoctadecanoate, methyl (±)-*trans*-9,10-epoxyoctadecanoate, methyl (±)-*cis*-11,12-epoxyoctadecanoate, methyl (±)-*trans*-11,12-epoxyoctadecanoate, methyl (±)-*cis*-12,13-epoxyoctadecanoate, methyl (±)-*cis*-8,9-epoxyeicosanoate, and methyl (±)-*cis*-11,12-epoxyeicosanoate were prepared in the same way by epoxidation of the corresponding monounsaturated esters followed by purification by TLC. Methyl (±)-*cis*-9,10-epoxy-12(Z)-octadecenoate and methyl (±)-*cis*-12,13-epoxy-9(Z)-octadecenoate were prepared by epoxidation of methyl linoleate using 1.1 equivalents of *m*-chloroperbenzoic acid followed by separation of the regioisomers by TLC (11). Similar treatment of methyl α-linolenate afforded methyl (±)-*cis*-12,13-epoxy-9(Z),15(Z)-octadecadienoate plus a mixture of methyl (±)-*cis*-9,10-epoxy-12(Z),15(Z)-octadecadienoate and methyl (±)-*cis*-15,16-epoxy-9(Z),12(Z)-octadecadienoate. The epoxy alcohols methyl 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate and 9(*R*),10(*S*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate were obtained by incubation of 13(*S*)-HPOD with *V. faba* epoxygenase (13) followed by separation of the diastereomers by TLC as recently described (10). Methyl 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(Z)-octadecenoate and methyl 11(*S*),12(*S*)-epoxy-13(*S*)-hydroxy-9(Z)-octadecenoate were prepared by intermolecular epoxidation of the methyl ester of 13(*S*)-HPOD in the presence of vanadium oxyacetylacetonate in hexane (9). Cumene hydroperoxide and sodium periodate were purchased from Sigma.

Enzyme Preparation and Incubation

Seeds of *V. faba* L., var Hangdown (Weibull Trädgård AB, Landskrona, Sweden) were ground in an electric coffee mill and the powder (20 g) was homogenized in 0.1 M potassium phosphate buffer, pH 6.7 (200 mL) at 0°C with a Polytron. The homogenate was filtered through cheesecloth and centrifuged at 9,300*g* for 15 min. The supernatant was further centrifuged at 269,000*g* for 1 h to provide a high-speed supernatant fraction (10 mg protein/mL) and a high-speed particle fraction. The particle fraction was resuspended in buffer and resedimented by centrifugation at 269,000*g* for 1 h. Suspensions of the washed particle fraction in buffer (2.5 mg protein/mL) were preincubated for 5 min at 22°C with 50 μM ETYA. Fatty acid substrate (100–300 μM) was added and the reaction was initiated by addition of either hydrogen peroxide (0.5–10 mM) or an organic hydroperoxide (200–600 μM). After stirring for 15 s to 10 min at 22°C, 5 volumes of methanol were added and the mixture was acidified to pH 3 to 4 and extracted with diethyl ether. The isolated material was treated with diazomethane and subsequently subjected to TLC using the solvent systems indicated. Recovery of radioactivity following incubation of ^{14}C-labeled substrates was about 95%.

Chemical Methods

Catalytic hydrogenation was performed by stirring the sample (0.01–5 mg) with 25 mg of 5% palladium on calcium carbonate (E. Merck, Darmstadt, Germany) in 3 mL of methanol under hydrogen gas for 1 h. This procedure resulted in a much lower extent of hydrogenolysis of allylic alcohols compared with that observed using procedures utilizing more active catalysts such as platinum oxide and palladium on carbon. Hydrolysis of epoxyesters into diols was carried out by treatment with 70% perchloric acid (0.15 mL) in dimethoxyethane (0.5 mL) and water (0.5 mL) at 22°C for 1 h. Alternatively, epoxyesters were converted into diols by treatment with formic acid (1 mL) at 22°C for 2 h followed by saponification of the hydroxy formate esters. Determination of the absolute configuration of epoxyesters was performed using a method previously described (6). Regio- and stereochemical analysis of trihydroxyoctadecenoic acids derived from epoxy alcohols was carried out as recently described (10).

Chromatographic and Instrumental Methods

TLC was carried out with precoated plates (kieselgel 60, 0.25 mm) from E. Merck. Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II interfaced with a Macintosh SE/30 PC. GLC was performed with a Hewlett-Packard model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. Peak areas were calculated using a Hewlett-Packard model 3396A integrator. GC-MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. Radioactivity was measured with a Packard Tri-Carb model 4450 liquid scintillation counter.

Assay of Fatty Acid Epoxidation

The ability of the *V. faba* epoxygenase to catalyze conversion of various monounsaturated fatty acids into their corresponding epoxide derivatives was determined by stirring the fatty acid (300 μM) with enzyme preparation (2 mL; preincubated for 5 min with 50 μM ETYA) at 22°C. The reaction was initiated by addition of hydrogen peroxide (5 mM) and quenched after 30 s by addition of methanol (10 mL) con-

taining (±)-*cis*-10,11-epoxynonadecanoic acid (126 μg). The esterified product was subjected to catalytic hydrogenation to convert the methyl ester of ETYA, which interfered with the gas-liquid chromatographic determination of epoxyesters, to methyl eicosanoate. The product was subjected to TLC (solvent system, ethyl acetate:hexane [1:9, v/v]) and the zone of epoxyesters was visualized by spraying with 2′,7′-dichlorofluorescein and viewing under UV. Material was eluted from the silica gel with diethyl ether and analyzed by GLC. The amount of epoxy acid formed from the fatty acid incubated was calculated from the peak areas of the chromatogram and from the amount of added epoxynonadecanoate. Because the reaction progress was essentially linear during the first 30 s of incubation (Fig. 1B), the results were expressed as the initial rate of formation of epoxy acid (nmol/s·mg protein).

In another set of experiments, mixtures of a monounsaturated fatty acid (150 μM) and oleic acid (150 μM) were stirred with the enzyme preparation for 30 s at 22°C in the presence of 5 mM hydrogen peroxide. The epoxyester fraction was isolated by TLC as described above and the ratio between the amounts of the two epoxyesters was determined by GLC.

RESULTS

Epoxidation of Oleic Acid

Incubation of [1-^{14}C]oleic acid (300 μM) with the enzyme preparation in the presence of either 13(*S*)-HPOD (400 μM) or cumene hydroperoxide (600 μM) resulted in the formation of a single oxygenated product of oleic acid, *i.e. cis*-9,10-epoxyoctadecanoic acid (*cf.* ref. 13). In addition, hydrogen peroxide efficiently supported the epoxidation. Figure 1 shows the hydrogen peroxide dependency and the time-course of the epoxidation of oleic acid. As seen, the percentage conversion of oleic acid at 30 s of incubation increased almost linearly when the hydrogen peroxide concentration

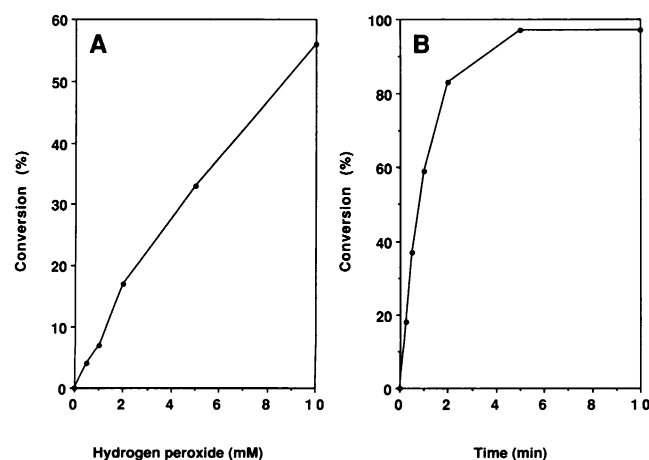


Figure 1. Percentage conversion of [1-^{14}C]oleic acid (300 μM) into *cis*-9,10-epoxyoctadecanoic acid in the presence of *V. faba* epoxygenase as a function of increasing concentration of hydrogen peroxide (A; time of incubation, 30 s) and time (B; concentration of hydrogen peroxide, 5 mM).

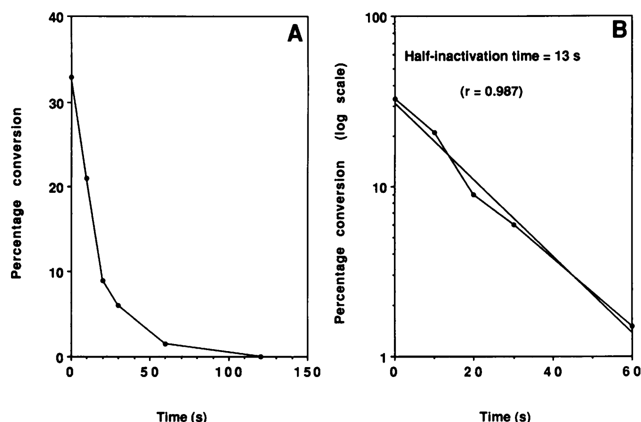


Figure 2. Inactivation of *V. faba* epoxygenase by hydrogen peroxide. The enzyme preparation was preincubated at 22°C for times indicated with 5 mM hydrogen peroxide and subsequently stirred with [1-^{14}C]oleic acid (300 μM) for 30 s. The percentage conversion (A, linear scale; B, log scale) into *cis*-9,10-epoxyoctadecanoic acid was determined by radio-TLC.

was increased from 0.5 to 10 mM (Fig. 1A). The epoxidation reaction was rapid. Thus, conversion of the added oleic acid was complete within 5 min of incubation using 5 mM hydrogen peroxide (Fig. 1B). The conversion was abolished when heat-inactivated (95°C, 10 min) enzyme preparation was used. Likewise, no conversion of [1-^{14}C]oleic acid was detectable in the presence of 0.5 to 10 mM hydrogen peroxide when enzyme was omitted.

The epoxygenase was readily inactivated by hydrogen peroxide when substrate was not present. As seen in Figure 2, the time needed to inactivate half of the enzyme activity using 5 mM hydrogen peroxide was about 13 s. Similar inactivation of epoxygenase in the presence of 13(*S*)-HPOD has been observed (13). The time course (Fig. 1B) that showed appreciable epoxidation during the period 1 to 5 min, coupled with the very low epoxygenase activity during this time period measured in the absence of substrate (Fig. 2), suggested that the fatty acid substrate protected the enzyme against inactivation.

Sodium periodate, an agent that has previously been shown to efficiently support cytochrome P-450-catalyzed hydroxylation of a number of steroids (14), did not serve as oxygen donor in the *V. faba* system when used in concentrations of 1 to 10 mM.

9,10-Epoxyoctadecanoate biosynthesized from oleic acid in the presence of either hydrogen peroxide, 13(*S*)-HPOD, or cumene hydroperoxide was subjected to steric analysis. In each case, the epoxy acid was mainly due to the 9(*R*),10(*S*) enantiomer, *i.e.* 81% 9(*R*),10(*S*) and 19% 9(*S*),10(*R*) (oxygen donor, 5 mM hydrogen peroxide); 80% 9(*R*),10(*S*) and 20% 9(*S*),10(*R*) (400 μM 13(*S*)-HPOD); and 82% 9(*R*),10(*S*) and 18% 9(*S*),10(*R*) (600 μM cumene hydroperoxide).

Monounsaturated Fatty Acids as Substrates for Epoxygenase

A series of monounsaturated fatty acids with chain lengths from 12 to 20 carbons and varying double bond positions

Table I. *Epoxidation of Monounsaturated Fatty Acids by V. faba Epoxygenase*

Fatty Acid	Product	C Value of Product	Initial Rate of Epoxidation[a]	Relative Rate of Epoxidation[b]
9(*Z*)-Dodecenoic acid	*cis*-9,10-Epoxydodecanoic acid	N.D.[c]	N.D.	N.D.
9(*E*)-Dodecenoic acid			0 (0)	N.D.
9(*Z*)-Tetradecenoic acid	*cis*-9,10-Epoxytetradecanoic acid	15.67	1.66 ± 0.10 (102)	87
9(*Z*)-Hexadecenoic acid	*cis*-9,10-Epoxyhexadecanoic acid	17.58	1.90 ± 0.07 (117)	122
6(*Z*)-Octadecenoic acid	*cis*-6,7-Epoxyoctadecanoic acid	19.53	0.54 ± 0.06 (33)	N.D.
6(*E*)-Octadecenoic acid	*trans*-6,7-Epoxyoctadecanoic acid	19.37	0.04 ± 0.006 (2)	1
9(*Z*)-Octadecenoic acid	*cis*-9,10-Epoxyoctadecanoic acid	19.55	1.62 ± 0.06 (100)	100
9(*E*)-Octadecenoic acid	*trans*-9,10-Epoxyoctadecanoic acid	19.42	0.06 ± 0.002 (4)	2
11(*Z*)-Octadecenoic acid	*cis*-11,12-Epoxyoctadecanoic acid	19.59	1.43 ± 0.05 (88)	N.D.
11(*E*)-Octadecenoic acid	*trans*-11,12-Epoxyoctadecanoic acid	19.44	0.04 ± 0.006 (2)	1
12(*Z*)-Octadecenoic acid	*cis*-12,13-Epoxyoctadecanoic acid	19.61	N.D.	N.D.
8(*Z*)-Eicosenoic acid	*cis*-8,9-Epoxyeicosanoic acid	21.53	0.67 ± 0.06 (41)	29
11(*Z*)-Eicosenoic acid	*cis*-11,12-Epoxyeicosanoic acid	21.55	0.81 ± 0.05 (50)	36

[a] Fatty acids (300 μM) were stirred at 22°C for 30 s with enzyme preparation (2.5 mg/mL protein; preincubated for 5 min with 50 μM ETYA) and hydrogen peroxide (5 mM). Methanol (5 volumes) containing (±)-*cis*-10,11-epoxynonadecanoic acid internal standard (126 μg) was added and the epoxyester fraction isolated by TLC was analyzed by GLC. Results are mean ± SD found in three experiments. Numbers within parentheses indicate rate relative to that of 9(*Z*)-octadecenoic acid. [b] Fatty acids (150 μM) plus 9(*Z*)-octadecenoic acid (150 μM) were stirred at 22°C for 30 s with enzyme preparation (2.5 mg/mL protein; preincubated for 5 min with 50 μM ETYA) and hydrogen peroxide (5 mM). Methanol (5 volumes) was added and the epoxyester fraction isolated by TLC was analyzed by GLC. Results are mean of two experiments. [c] ND, Not determined.

and configurations (Table I) were incubated for 30 s with the *V. faba* epoxygenase in the presence of 5 mM hydrogen peroxide. The resulting epoxy acids were isolated as their methyl esters and their identities ascertained by GLC and GC-MS analysis. Authentic epoxyesters prepared by chemical epoxidation were used as references. The identities of the enzymically produced epoxyesters were further proved by mass-spectrometric analysis of the Me_3Si derivatives of the corresponding dihydroxyesters obtained following perchloric acid-catalyzed hydrolysis (*cf.* ref. 13). The initial rate of production of epoxy acids was determined by GLC using (±)-*cis*-10,11-epoxynonadecanoic acid as internal standard. As seen in Table I, 9(Z)-hexadecenoic acid served as the best substrate followed by 9(Z)-tetradecenoic, 9(Z)-octadecenoic, 11(Z)-octadecenoic, 11(Z)-eicosenoic, 8(Z)-eicosenoic, and 6(Z)-octadecenoic acids. The *trans* fatty acids 9(*E*)-, 11(*E*)-, and 6(*E*)-octadecenoic acids were converted at a very slow rate to provide the corresponding *trans* epoxide derivatives. In the case of 9(*E*)-dodecenoic acid, no conversion (<1%) could be detected.

Results of experiments in which mixtures of a monounsaturated fatty acid and oleic acid (both 150 μM) were incubated are also shown in Table I. As seen, the ranking order of effective substrates found using this assay was essentially the same as that found in the single substrate incubations.

The absolute configurations of epoxyesters obtained following incubation of a number of (Z)-monoenoic acids are given in Table II. As seen, the majority of the fatty acids examined yielded epoxide derivatives that had mainly the (*R*),(*S*) configuration. 12(Z)-Octadecenoic acids produced an epoxide that was mainly due to the (*S*),(*R*) enantiomer, whereas 11(Z)-octadecenoic acid produced an epoxide that was nearly racemic.

Linoleic Acid as Substrate for Epoxygenase

[1-^{14}C]Linoleic acid (300 μM) was stirred at 22°C for 1 and 10 min with enzyme preparation (5 mL, 12.5 mg protein; preincubated for 5 min with 50 μM ETYA) and hydrogen peroxide (5 mM). The esterified product was analyzed by radio-TLC. Four compounds, *i.e.* methyl 9,10:12,13-diepoxyoctadecanoate (compound A, R_F = 0.13–0.17), methyl *cis*-

Table II. *Enantiomeric Composition of Epoxy Acids Formed from Z-Monoenoic Acids*

Substrate	Product	Enantiomeric Composition
9(Z)-Dodecenoic acid	*cis*-9,10-Epoxydodecanoic acid	94% 9(*R*),10(*S*); 6% 9(*S*),10(*R*)
9(Z)-Tetradecenoic acid	*cis*-9,10-Epoxytetradecanoic acid	55% 9(*R*),10(*S*); 45% 9(*S*),10(*R*)
9(Z)-Hexadecenoic acid	*cis*-9,10-Epoxyhexadecanoic acid	71% 9(*R*),10(*S*); 29% 9(*S*),10(*R*)
6(Z)-Octadecenoic acid	*cis*-6,7-Epoxyoctadecanoic acid	66% 6(*R*),7(*S*); 34% 6(*S*),7(*R*)
9(Z)-Octadecenoic acid	*cis*-9,10-Epoxyoctadecanoic acid	81% 9(*R*),10(*S*); 19% 9(*S*),10(*R*)
11(Z)-Octadecenoic acid	*cis*-11,12-Epoxyoctadecanoic acid	53% 11(*S*),12(*R*); 47% 11(*R*),12(*S*)
12(Z)-Octadecenoic acid	*cis*-12,13-Epoxyoctadecanoic acid	64% 12(*S*),13(*R*); 36% 12(*R*),13(*S*)
8(Z)-Eicosenoic acid	*cis*-8,9-Epoxyeicosanoic acid	59% 8(*R*),9(*S*); 41% 8(*S*),9(*R*)
11(Z)-Eicosenoic acid	*cis*-11,12-Epoxyeicosanoic acid	61% 11(*R*),12(*S*); 39% 11(*S*),12(*R*)

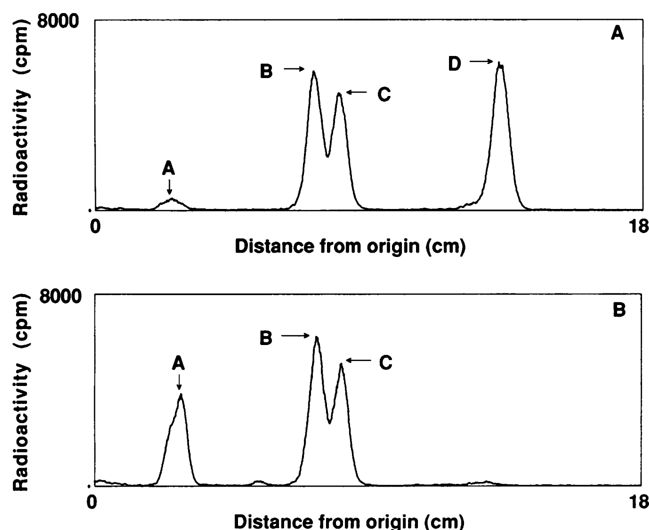


Figure 3. Thin-layer radiochromatograms of esterified products obtained following incubation of [1-^{14}C]linoleic acid (300 μM) with *V. faba* epoxygenase and hydrogen peroxide for 1 min (A) and 10 min (B). Peak A, Methyl 9,10:12,13-diepoxyoctadecanoate; peak B, methyl *cis*-9,10-epoxy-12(*Z*)-octadecenoate; peak C, methyl *cis*-12,13-epoxy-9(*Z*)-octadecenoate; peak D, methyl linoleate. Solvent system, ethyl acetate:hexane (1:9, v/v).

9,10-epoxy-12(Z)-octadecenoate (compound B, R_F = 0.40), methyl *cis*-12,13-epoxy-9(Z)-octadecenoate (compound C, R_F = 0.45), and methyl linoleate (compound D, corresponding to linoleic acid remaining unconverted) were observed (Fig. 3). Compounds B and C, the *cis*-monoepoxide derivatives of methyl linoleate, were identified by GC-MS as described in detail (13). Furthermore, catalytic hydrogenation followed by perchloric acid-catalyzed hydrolysis performed on compounds B and C provided methyl *threo*-9,10-dihydroxyoctadecanoate and methyl *threo*-12,13-dihydroxyoctadecanoate, respectively (13). Compound A, the diepoxide derivative of methyl linoleate, separated into two diastereomers upon GLC analysis (ratio, approximately 1:1; C values, 20.83 and 21.12). The mass spectra of the two diastereomers were virtually identical and showed prominent ions at m/z 308 (M-18; loss of H_2O), 277 (M-(18 + 31); loss of H_2O plus $\cdot OCH_3$), 187 ($HO^+ = CH\text{-}(CH_2)_7\text{-}COOCH_3$), and 155 (187–32; loss of CH_3OH). Further support for the presence in compound A of epoxide groups at C-9,10 and C-12,13 was provided by the finding that compound A (100 μg) upon treatment with formic acid followed by saponification and reesterification yielded methyl 9,10,12,13-tetrahydroxyoctadecanoate. Analysis of the Me_3Si derivative by GC-MS showed the presence of two diastereomers (ratio, approximately 1:1; C-23.99 and 24.06), the mass spectra of which were identical to those of authentic material (13).

As seen in Figure 3, incubation of linoleic acid for either 1 or 10 min led to the formation of monoepoxyoctadecenoates as the main products (yields at 1 and 10 min, 57 and 69%, respectively; ratio methyl 9,10-epoxyoctadecenoate to methyl 12,13-epoxyoctadecenoate, 55:45). In contrast, the diepoxyoctadecanoate was obtained in low yield (3%) at 1 min incubation and in a higher yield (26%) following incubation for 10 min. Apparently, linoleic acid underwent a rapid epoxidation at the Δ^9 and Δ^{12} double bonds to produce monoepoxide derivatives. These compounds were slowly further epoxidized into the diepoxyoctadecanoate.

Steric analysis showed that the methyl *cis*-9,10-epoxy-12(Z)-octadecenoate (compound B) obtained from linoleic acid following a 1-min incubation with epoxygenase and hydrogen peroxide was due to 83% of the 9(*R*),10(*S*) enantiomer and that the methyl *cis*-12,13-epoxyoctadecenoate (compound C) was due to 64% of the 12(*S*),13(*R*) enantiomer (Table III). These enantiomeric compositions were similar to those found for 9,10- and 12,13-epoxyoctadecenoates produced from linoleic acid with 13(*S*)-HPOD as the oxygen donor (13). Steric analysis of the two epoxides produced from linoleic acid in a 10-min incubation showed a slight increase in optical purity, *i.e.* *cis*-9,10-epoxyoctadecenoate, 87% 9(*R*),10(*S*) enantiomer, and *cis*-12,13-epoxyoctadecenoate, 72% 12(*S*),13(*R*) enantiomer (Table III). Thus, it appeared that there was a preference in the utilization of 9(*S*),10(*R*)-epoxy-12(Z)-ocadecenoic acid and 12(*R*),13(*S*)-epoxy-9(Z)-octadecenoic acid compared with their corresponding enantiomers in the second epoxidation reaction.

α-Linolenic Acid as Substrate for Epoxygenase

Analysis of the product obtained following incubation of [1-^{14}C]α-linolenic acid (300 μM) with enzyme preparation and hydrogen peroxide (5 mM) at 22°C for 1 min showed the presence of methyl diepoxyoctadecenoates (6%, R_F = 0.12–

Table III. *Enantiomeric Composition of Monoepoxy Acids Formed from Linoleic and α-Linolenic Acids*

Substrate	Incubation Time	Product	Enantiomeric Composition
Linoleic acid	1 min	*cis*-9,10-Epoxy-12(*Z*)-octadecenoic acid	83% 9(*R*),10(*S*); 17% 9(*S*),10(*R*)
		cis-12,13-Epoxy-9(*Z*)-octadecenoic acid	64% 12(*S*),13(*R*); 36% 12(*R*),13(*S*)
Linoleic acid	10 min	*cis*-9,10-Epoxy-12(*Z*)-octadecenoic acid	87% 9(*R*),10(*S*); 13% 9(*S*),10(*R*)
		cis-12,13-Epoxy-9(*Z*)-octadecenoic acid	72% 12(*S*),13(*R*); 28% 12(*R*),13(*S*)
α-Linolenic acid	1 min	*cis*-9,10-Epoxy-12(*Z*),15(*Z*)-octadecadienoic acid	73% 9(*R*),10(*S*); 27% 9(*S*),10(*R*)
		cis-12,13-Epoxy-9(*Z*),15(*Z*)-octadecadienoic acid	73% 12(*S*),13(*R*); 27% 12(*R*),13(*S*)
		cis-15,16-Epoxy-9(*Z*),12(*Z*)-octadecadienoic acid	69% 15(*R*),16(*S*); 31% 15(*S*),16(*R*)
α-Linolenic acid	10 min	*cis*-9,10-Epoxy-12(*Z*),15(*Z*)-octadecadienoic acid	80% 9(*R*),10(*S*); 20% 9(*S*),10(*R*)
		cis-12,13-Epoxy-9(*Z*),15(*Z*)-octadecadienoic acid	80% 12(*S*),13(*R*); 20% 12(*R*),13(*S*)
		cis-15,16-Epoxy-9(*Z*),12(*Z*)-octadecadienoic acid	68% 15(*R*),16(*S*); 32% 15(*S*),16(*R*)

0.16), methyl *cis*-9,10-epoxy-12(Z),15(Z)-octadecadienoate plus methyl *cis*-15,16-epoxy-9(Z),12(Z)-octadecadienoate (48% and 5%, respectively, $R_F = 0.38$–0.39), and methyl *cis*-12,13-epoxy-9(Z),15(Z)-octadecadienoate (29%, $R_F = 0.45$). Similar incubation of α-linolenic acid carried out for 10 min led to the formation of the same products, although the yield of diepoxyoctadecenoates was higher (26%).

Identification of the monoepoxyoctadecadienoates was made by GC-MS using the authentic compounds as references. The *cis*-9,10- and *cis*-15,16-epoxyoctadecadienoates cochromatographed upon TLC and GLC analysis. Determination of the individual isomers was carried out by GLC following catalytic hydrogenation into *cis*-9,10- and *cis*-15,16-octadecanoates (C values, 19.55 and 19.78, respectively). Further proof for the identity and percentage amounts of the monoepoxyoctadecadienoates was obtained by GC-MS analysis of the Me_3Si derivatives of the diol esters obtained following catalytic hydrogenation and perchloric acid-catalyzed hydrolysis, *i.e.* methyl *threo*-9,10-dihydroxyoctadecanoate (C-21.25), methyl *threo*-12,13-dihydroxyoctadecanoate (C-21.48), and methyl *threo*-15,16-dihydroxyoctadecanoate (C-22.38).

Analysis of the methyl diepoxyoctadecenoate fraction by GLC showed three isomers (C-20.82, C-21.03, and C-21.09), the mass spectra of which showed ions at *inter alia* m/z 295 (M-29; loss of $\cdot C_2H_5$), 277 (M-(29 + 18); loss of $\cdot C_2H_5$ plus H_2O), 187 ($HO^+ = CH\text{-}(CH_2)_7\text{-}COOCH_3$), and 155 (187–32; loss of CH_3OH). Treatment of material present in the diepoxyoctadecenoate fraction with formic acid followed by sodium hydroxide and reesterification yielded a mixture of regio- and diastereomeric methyl tetrahydroxyoctadecenoates. This material was not examined in detail; however, analysis of the Me_3Si derivatives by GC-MS showed the presence mainly of diastereomers of methyl 9,10,12,13-tetrahydroxy-15-octadecenoate (ions *inter alia* at m/z 387 [M-(171 + 90); loss of $\cdot CH(OSiMe_3)\text{-}CH_2\text{-}CH = CH\text{-}C_2H_5$ plus Me_3SiOH] and m/z 361 [$Me_3SiO^+ = CH\text{-}CH(OSiMe_3)\text{-}(CH_2)_7\text{-}COOCH_3$]). Smaller amounts of diastereomers of methyl 9,10,15,16-tetrahydroxy-12-octadecenoate were also observed (ions *inter alia* at m/z 427 [M-(131 + 90); loss of $\cdot CH(OSiMe_3) - C_2H_5$ plus Me_3SiOH] and m/z 361 [$Me_3SiO^+ = CH\text{-}CH(OSiMe_3)\text{-}(CH_2)_7\text{-}COOCH_3$]).

Results of steric analyses of monoepoxyoctadecadienoates produced from α-linolenic acid upon incubation for 1 min with epoxygenase are given in Table III. In line with the results found with linoleic acid, the 9,10-epoxyoctadecadienoate was mainly due to the 9(*R*),10(*S*) enantiomer and the 12,13-epoxyoctadecadienoate was mainly the 12(*S*),13(*R*) enantiomer. Furthermore, extending the time of incubation of α-linolenic acid from 1 to 10 min resulted in the formation of 9,10- and 12,13-epoxyoctadecadienoates with a slightly higher optical purity, whereas that of the 15,16-epoxyoctadecadienoate remained unchanged (Table III). Thus, as found with linoleic acid, there was a preference in the utilization of the 9(*S*),10(*R*)- and 12(*R*),13(*S*)-monoepoxides compared with their corresponding enantiomers in the second step of epoxidation that produced diepoxides.

13(*S*)-HOD as Substrate for Epoxygenase

13(*S*)-[1-^{14}C]HOD (300 μM) was stirred with enzyme preparation (10 mL, 25 mg protein) and hydrogen peroxide (5 mM) for 10 min at 22°C. The aqueous phase was acidified to pH 4 and rapidly extracted with two volumes of diethyl ether. As seen in Figure 4, analysis of the esterified product by radio-TLC (solvent, ethyl acetate: hexane [3:7, v/v]) showed the presence of the methyl ester of 13(*S*)-HOD (26%, $R_F = 0.67$) as well as two more polar compounds, Compound A (66%, $R_F = 0.40$) and compound B (4%, $R_F = 0.52$). The last mentioned compound cochromatographed with the α,β-epoxy alcohol methyl 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(Z)-octadecenoate prepared by vanadium oxyacetylacetonate-catalyzed epoxidation of the methyl ester of 13(*S*)-HPOD (9). Furthermore, the mass spectrum recorded on the Me_3Si derivative of compound B was identical to that of the chemically prepared epoxy alcohol. Final proof for the identity of compound B with methyl 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(Z)-octadecenoate (Fig. 5) was provided by an experiment in which compound B (200 μg in 0.5 mL methanol) was hydrolyzed by treatment with 50 mL acidified water (pH 3) at 22°C for 5 min (*cf.* refs. 9 and 10). This treatment yielded isomeric trihydroxyoctadecenoates, which were, as expected (9), mainly due to a 1:1 mixture of methyl 9(*R*),12(*S*),13(*S*)- and 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoates.

Compound A obtained following incubation of 13(*S*)-HOD separated into two compounds, compounds A_1 ($R_F = 0.44$, 87%) and A_2 ($R_F = 0.48$, 13%), when subjected to TLC using ethyl acetate:toluene (3:7, v/v), as the solvent system (references, methyl 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate [$R_F = 0.44$] and methyl 9(*R*),10(*S*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate [$R_F = 0.48$]). The mass spectra of the Me_3Si derivatives of compounds A_1 and A_2 were identical with those of the Me_3Si derivatives of the 9(*S*),10(*R*)- and 9(*R*),10(*S*)-epoxy alcohols, respectively (13). Oxidative ozonolysis performed on the (−)-menthoxycarbonyl derivatives (8) of compounds A_1 and A_2 yielded the (−)-menthox-

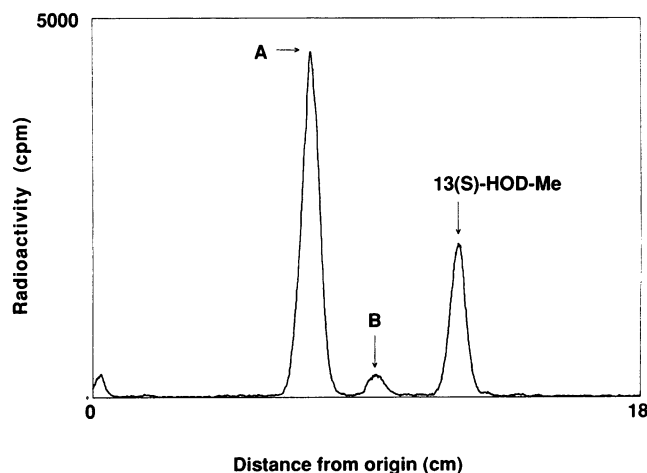


Figure 4. Thin layer radiochromatogram of esterified product obtained following incubation of 13(*S*)-[1-^{14}C]HOD (300 μM) with *V. faba* epoxygenase and hydrogen peroxide for 10 min. A, Mixture of methyl 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate and methyl 9(*R*),10(*S*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate (ratio, 87:13); B, methyl 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoate. Solvent system, ethyl acetate:hexane (3:7, v/v).

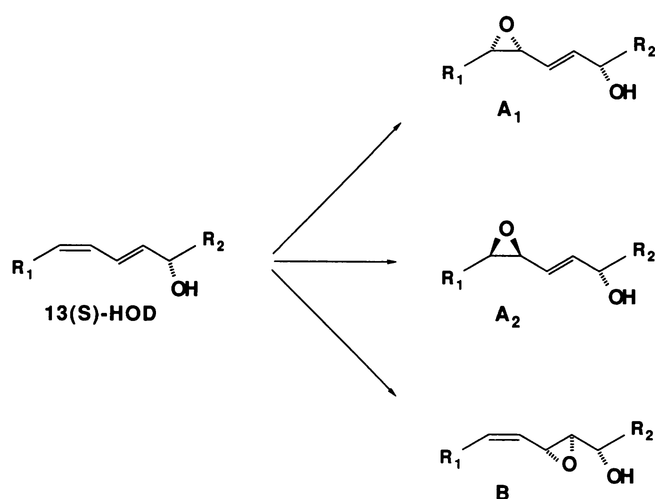


Figure 5. Structures of epoxy alcohols formed by enzymic epoxidation of 13(*S*)-HOD. R_1 = $(CH_2)_7$-COOH; R_2 = $(CH_2)_4$-CH_3.

ycarbonyl derivatives of 2(*S*)-hydroxyheptanoic acid, thus localizing the double bond to the Δ^{11} position and showing that the configuration at C-13 of both compounds was (*S*). Further proof for the identities of compounds A_1 and A_2 with methyl 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate and methyl 9(*R*),10(*S*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoate, respectively (Fig. 5), came from steric analysis of trihydroxyoctadecenoates obtained following acid-catalyzed hydrolysis as described in detail (10). As expected (10), hydrolysis of compound A_1 afforded four isomeric trihydroxyoctadecenoates of which methyl 9(*S*),10(*S*),13(*S*)-trihydroxy-11(*E*)-octadecenoate was the major one (61% of total trihydroxyoctadecenoates). Similarly, the major trihydroxyester obtained from compound A_2 was identified as methyl 9(*R*),10(*R*),13(*S*)-trihydroxy-11(*E*)-octadecenoate.

Epoxide Hydrolase in *V. faba* Seeds

Incubation of [1-^{14}C]*cis*-9,10-epoxyoctadecanoic acid (150 μM) with the 269,000*g* supernatant fraction of homogenate of *V. faba* resulted in the formation of a more polar compound. As seen in Figure 6, the reaction was complete within 30 min. Heat treatment of the supernatant fraction abolished the conversion. Analysis of the methyl ester-Me_3Si derivative of the polar compound by GLC showed a C value of 21.25 (references, Me_3Si derivatives of methyl *erythro*- and *threo*-9,10-dihydroxyoctadecanoates, C-21.48 and C-21.25, respectively). The mass spectrum, which showed prominent ions at m/z 443 (M-31, loss of $\cdot OCH_3$), 332 (M-142, rearrangement followed by elimination of OHC-$(CH_2)_7$-CH_3), 259 (Me_3SiO^+ = CH-$(CH_2)_7$-$COOCH_3$), 215 (Me_3SiO^+ = CH-$(CH_2)_7$-CH_3), and 155 (OHC-$(CH_2)_7$-C $\equiv O^+$), was identical to that of the Me_3Si derivative of authentic methyl *threo*-9,10-dihydroxyoctadecanoate.

It was conceivable that one of the enantiomers of *cis*-9,10-epoxyoctadecanoic acid was preferentially utilized in the enzymic conversion into *threo*-9,10-dihydroxyoctadecanoic acid. To investigate this possibility, *cis*-9,10-epoxyoctadecanoic acid (ratio 9(*R*),10(*S*) to (9*S*),10(*R*) enantiomers, 82:18) was incubated for 10 min with the high-speed supernatant fraction (conversion, 42%) and the nonconverted epoxy acid reisolated. Steric analysis carried out on this material showed an enantiomeric composition of 9(*R*),10(*S*):9(*S*),10(*R*) = 71:29, demonstrating that the rate of hydrolysis of the 9(*R*),10(*S*) enantiomer was considerably higher than that of the 9(*S*),10(*R*) enantiomer.

DISCUSSION

Biosynthesis of a plant epoxy fatty acid in a cell-free system was first demonstrated by Croteau and Kolattukudy (3). These workers demonstrated that 18-hydroxyoleic acid was converted into 18-hydroxy-*cis*-9,10-epoxyoctadecanoic acid when incubated with a particulate preparation from spinach. The facts that NADPH and molecular oxygen were required and that the reaction was inhibited by carbon monoxide indicated that the epoxygenase was a cytochrome P-450 protein. Furthermore, the reaction had an absolute requirement for ATP and CoA, suggesting that 18-hydroxyoleoyl-CoA was the actual substrate. In a more recent study, Salaün *et al.* (22) demonstrated epoxidation of 9(*Z*)- and 9(*E*)-dodecenoic acids into *cis*- and *trans*-epoxydodecanoic acids, respectively, in the presence of a microsomal cytochrome P-450 from Jerusalem artichoke. A cytochrome P-450-independent pathway for plant epoxy acid biosynthesis was recently discovered by Hamberg and Hamberg (13) and by Blée and Schuber (1). This reaction, which was catalyzed by an epoxygenase present in the microsomal fraction of homogenate of seeds of *V. faba* (13) and by a partially purified peroxygenase obtained from microsomes of soybean seedlings (1), consisted of epoxidation of unsaturated fatty acids by a hydroperoxide-dependent mechanism. The *V. faba* epoxygenase and the soybean peroxygenase had many features

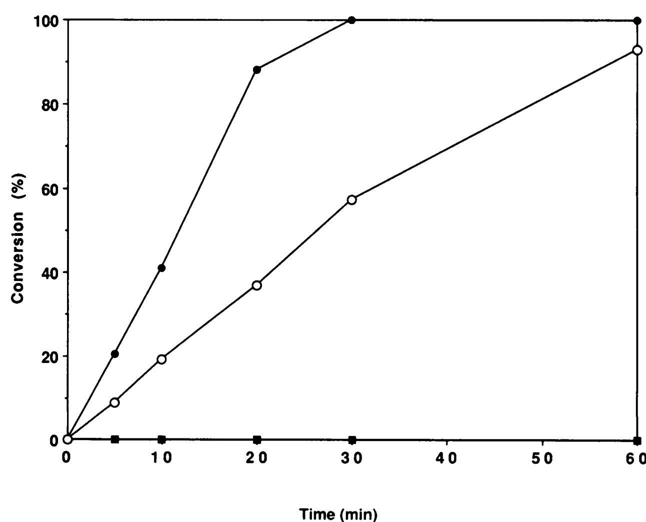


Figure 6. Percentage conversion of [1-^{14}C]*cis*-9,10-epoxyoctadecanoic acid into *threo*-9,10-dihydroxyoctadecanoic acid by *V. faba* epoxide hydrolase. ●, 150 μM epoxyoctadecanoic acid; O, 450 μM epoxyoctadecanoic acid; ■, 150 μM epoxyoctadecanoic acid and heat-treated enzyme.

in common, including the hydroperoxide dependency, the mechanism of epoxidation involving a direct transfer of oxygen from hydroperoxide to substrate, and the lack of inhibition by antioxidants and cytochrome P-450 inhibitors.

Hydrogen peroxide is a normal constituent of plant cells. Organelles that produce hydrogen peroxide include chloroplasts, mitochondria, microsomes, and peroxisomes. It has been estimated that the steady-state concentration of hydrogen peroxide in leaf cells is in the range 0.1 to 1 mM (21, 23). In the present study, we have found that hydrogen peroxide can serve as an effective oxygen donor in the *V. faba* epoxygenase reaction (Fig. 1). The product isolated following incubation of oleic acid with epoxygenase in the presence of hydrogen peroxide was identical with that previously obtained following incubation of oleic acid in the presence of 13(*S*)-HPOD and cumene hydroperoxide (13), *i.e. cis*-9,10-epoxyoctadecanoic acid. The stereoisomeric composition of the epoxide was found to be 80 to 82% of the 9(*R*),10(*S*) enantiomer and 18 to 20% of the 9(*S*),10(*R*) enantiomer, irrespective of the use of an organic hydroperoxide or hydrogen peroxide as the oxygen donor. Examination of the specificity of the *V. faba* epoxygenase in catalyzing hydrogen peroxide-supported epoxidation of a number of monounsaturated fatty acids showed that the rate of epoxidation was affected by the double bond position as well as by the chain length of the substrate (Table I). (*E*)-Fatty acids were poor substrates for the epoxygenase and produced a low yield of the corresponding *trans*-epoxy acids. Steric analysis of *cis*-epoxy fatty acids produced by epoxidation of a series of (Z)-monounsaturated fatty acids revealed a preponderance for the (*R*),(*S*) epoxides in most cases (Table II). Exceptions were *cis*-11,12-epoxyoctadecanoate, which was close to racemic, and *cis*-12,13-epoxyoctadecanoate, which was mainly due to the (*S*),(*R*) enantiomer. The highest optical purity was recorded for *cis*-9,10-epoxydodecanoic acid, *i.e.* 9(*R*),10(*S*):9(*S*),10(*R*), 94:6. This optical purity was higher than that determined for the same compound biosynthesized by cytochrome P-450-catalyzed epoxidation of 9(Z)-dodecenoic acid, *i.e.* 9(*R*),10(*S*):9(*S*),10(*R*), 78:22 (5).

Incubation of linoleic acid with *V. faba* epoxygenase for 1 min in the presence of hydrogen peroxide produced a mixture mainly consisting of *cis*-9,10-epoxy-12(Z)-octadecenoic acid and *cis*-12,13-epoxy-9(Z)-octadecenoic acid (Fig. 3A). The enantiomeric compositions of the two monoepoxides were essentially the same as those previously reported in the case of the 13(*S*)-HPOD-supported epoxidation of linoleic acid (13), *i.e.* the major enantiomers were 9(*R*),10(*S*)- and 12(*S*),13(*R*)-epoxyoctadecenoates (Table III). Incubation of linoleic acid for a 10-min period led to further epoxidation of the monoepoxides into 9,10:12,13-diepoxyoctadecanoate (Fig. 3B). Interestingly, in this second step of epoxidation the 9(*S*),10(*R*)- and 12(*R*),13(*S*)-epoxyoctadecenoic acids were epoxidized at a higher rate compared with their corresponding enantiomers as judged by steric analysis of the remaining monoepoxyoctadecenoates (Table III).

Also, α-linolenic acid was rapidly converted into epoxy acids in the presence of *V. faba* epoxygenase and hydrogen peroxide. The monoepoxyoctadecadienoic acids were due to *cis*-9,10-epoxy-12(Z),15(Z)-octadecadienoic acid (mainly 9(*R*),10(*S*)), *cis*-12,13-epoxy-9(Z),15(Z)-octadecadienoic acid (mainly 12(*S*),13(*R*)), and *cis*-15,16-epoxy-9(Z),12(Z)-octadecadienoic acid (mainly 15(*R*),16(*S*)) (Table III). As with linoleic acid, incubation for 10 min resulted in further epoxidation of the monoepoxy acids. The structures of the resulting diepoxyoctadecenoates were not studied in detail; however, mass spectrometric analysis of the tetrahydroxyoctadecenoates obtained following hydrolysis indicated that the diepoxyoctadecenoate fraction was mainly due to 9,10:12,13-diepoxy-15(Z)-octadecenoate plus a smaller amount of 9,10:15,16-diepoxy-12(Z)-octadecenoate. The *cis*-9,10- and *cis*-12,13-epoxyoctadecadienoates—but not the *cis*-15,16-epoxyoctadecadienoate—isolated following incubation of α-linolenic acid for a 10-min period were enriched with respect to the 9(*R*),10(*S*) and 12(*S*),13(*R*) enantiomers, respectively (Table III). Thus, as was the case with linoleic acid, there appeared to be a preference in epoxidizing the 9(*S*),10(*R*)- and 12(*R*),13(*S*)-monoepoxy acids.

Comparison of available data on the specificities of *V. faba* epoxygenase and soybean peroxygenase reveals a few important differences. Thus, in contrast to *V. faba* epoxygenase (Table I), soybean peroxygenase catalyzed epoxidation of regioisomeric (Z)-octadecenoic acids at the same rate (1). Also, in variance with the *V. faba* enzyme (Table I), the rates of epoxidation of 9(Z)-hexadecenoic and 9(Z)-tetradecenoic acids by the peroxygenase were markedly slower than that of 9(Z)-octadecenoic acid (1). Another notable difference between the two enzymes was the stereochemistry of *cis*-12,13-epoxy-9(Z)-octadecenoic acid biosynthesized from linoleic acid. *V. faba* epoxygenase produced mainly the 12(*S*),13(*R*)-epoxide (28% enantiomeric excess) (13, present work), whereas soybean peroxygenase produced mainly the 12(*R*),13(*S*)-epoxide (39% enantiomeric excess) (2).

Two diastereomeric epoxy alcohols, *i.e.* 9(*S*),10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid and 9(*R*),10(*S*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid (ratio, 91:9), were previously isolated following incubation of 13(*S*)-HPOD with *V. faba* epoxygenase (10, 13). Detailed studies using $^{18}O_2$-labeled 13(*S*)-HPOD showed that two pathways were responsible for this conversion, *i.e.* intramolecular epoxidation of the Δ^9 double bond by the distal hydroperoxide oxygen of 13(*S*)-HPOD, as well as an intermolecular reaction in which the Δ^9 double bond of 13(*S*)-HOD was epoxidized by hydroperoxide oxygen of 13(*S*)-HPOD. In the present study, incubation of 13(*S*)-HOD with epoxygenase and hydrogen peroxide led to the formation of the above-mentioned epoxy alcohols (ratio 9(*S*),10(*R*)- to 9(*R*),10(*S*)-epoxides, 87:13). In addition, a minor epoxy alcohol identified as 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(Z)-octadecenoic acid was obtained (Fig. 5). The preponderance of the two diastereomeric 9,10-epoxy alcohols over the 11,12-epoxy alcohol was probably explained by the large rate difference in the epoxidation of (Z) *versus* (*E*) double bonds (*cf.* Table I).

Further studies are needed to assess the biological role of the hydroperoxide-dependent epoxygenase pathway in plant tissue. In this context, it should be mentioned that Kato *et al.* has isolated an array of oxygenated fatty acids from rice plant in response to plant pathogens (15, 17). A number of these compounds exert potent antifungal effects (18). Such compounds include 9,10- and 12,13-epoxyoctadecenoic acids as well as 9,10-, 12,13-, and 15,16-epoxyoctadecadienoic acids.

Trihydroxyoctadecenoic and trihydroxyoctadecadienoic acids constitute another series of antifungal compounds isolated from rice in rice blast disease (16) and from tubers of taro inoculated with black root fungus (19). The hydroperoxide-dependent epoxygenase pathway offers a mechanism for biosynthesis of such plant defensive substances. For example, the five above-mentioned monoepoxyoctadecenoic and monoepoxyoctadecadienoic acids are products of linoleic and α-linolenic acid epoxidation (Table III). Furthermore, isomeric trihydroxyoctadecenoic and trihydroxyoctadecadienoic acid are produced by hydrolysis of allylic epoxy alcohols (10), which are in turn formed by epoxidation of hydroxy acids (Fig. 5) or by intramolecular epoxidation of fatty acid hydroperoxides (13). Recently, Ohta *et al.* (20) reported that rice seeds contain a lipid-hydroperoxide-decomposing activity that catalyzed the conversion of 9-hydroperoxy-10,12-octadecadienoic acid into a mixture of 9-hydroxy-10,12-octadecadienoic acid and 9,12,13-trihydroxy-10-octadecenoic acid, both having an antifungal effect. This hydroperoxide-decomposing activity may well be a hydroperoxide-dependent epoxygenase similar to the *V. faba* epoxygenase and the soybean peroxygenase.

ACKNOWLEDGMENT

We wish to thank Gunvor Hamberg for expert technical assistance.

LITERATURE CITED

1. **Blée E, Schuber F** (1990) Efficient epoxidation of unsaturated fatty acids by a hydroperoxide-dependent oxygenase. J Biol Chem **265:** 12887–12894
2. **Blée E, Schuber F** (1990) Stereochemistry of the epoxidation of fatty acids catalyzed by soybean peroxygenase. Biochem Biophys Res Commun **173:** 1354–1360
3. **Croteau R, Kolattukudy PE** (1975) Enzymatic epoxidation of 18-hydroxyoleic acid to 18-hydroxy-*cis*-9,10-epoxystearic acid by a particulate preparation from spinach (*Spinacia oleracea*). Arch Biochem Biophys **170:** 61–72
4. **Fahlstadius P** (1988) *cis*-9,10-Epoxystearic acid in human leukocytes: isolation and quantitative determination. Lipids **23:** 1015–1018
5. **Fahlstadius P** (1991) Absolute configurations of 9,10-epoxydodecanoic acids biosynthesized by microsomes from Jerusalem artichoke tubers. Phytochemistry **30:** 1905–1907
6. **Fahlstadius P, Hamberg M** (1989) A gas-liquid chromatographic method for steric analysis of epoxy acids. Chem Phys Lipids **51:** 15–22
7. **Gunstone FD, Jacobsberg FR** (1972) The preparation and properties of the complete series of methyl epoxyoctadecanoates. Chem Phys Lipids **9:** 26–34
8. **Hamberg M** (1971) Steric analysis of hydroperoxides formed by lipoxygenase oxygenation of linoleic acid. Anal Biochem **43:** 515–526
9. **Hamberg M** (1987) Vanadium-catalyzed transformation of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid: structural studies on epoxy alcohols and trihydroxy acids. Chem Phys Lipids **43:** 55–67
10. **Hamberg M** (1991) Regio- and stereochemical analysis of trihydroxyoctadecenoic acids derived from linoleic acid 9- and 13-hydroperoxides. Lipids **26:** 407–415
11. **Hamberg M, Fahlstadius P** (1990) Allene oxide cyclase: a new enzyme in plant lipid metabolism. Arch Biochem Biophys **276:** 518–526
12. **Hamberg M, Gotthammar B** (1973) A new reaction of unsaturated fatty acid hydroperoxides: formation of 11-hydroxy-12,13-epoxy-9-octadecenoic acid from 13-hydroperoxy-9,11-octadecadienoic acid. Lipids **8:** 737–744
13. **Hamberg M, Hamberg G** (1990) Hydroperoxide-dependent epoxidation of unsaturated fatty acids in the broad bean (*Vicia faba* L.). Arch Biochem Biophys **283:** 409–416
14. **Hrycay EG, Gustafsson JÅ, Ingelman-Sundberg M, Ernster L** (1975) Sodium periodate, sodium chlorite, organic hydroperoxides, and H_2O_2 as hydroxylating agents in steroid hydroxylation reactions catalyzed by partially purified cytochrome P-450. Biochem Biophys Res Commun **66:** 209–216
15. **Kato T, Yamaguchi Y, Hirano T, Yokoyama T, Uyehara T, Namai T, Yamanaka S, Harada N** (1984) Unsaturated hydroxy fatty acids, the self defensive substances in rice plant against rice blast disease. Chem Lett 409–412
16. **Kato T, Yamaguchi Y, Hirukawa T, Hoshino N** (1991) Structural elucidation of naturally occurring 9,12,13-trihydroxy fatty acids by a synthetic study. Agric Biol Chem **55:** 1349–1357
17. **Kato T, Yamaguchi Y, Uyehara T, Yokoyama T, Namai T, Yamanaka S** (1983) Self defensive substances in rice plant against rice blast disease. Tetrahedron Lett **24:** 4715–4718
18. **Kato T, Yamaguchi Y, Uyehara T, Yokoyama T, Namai T, Yamanaka S** (1983) Defense mechanism of the rice plant against rice blast disease. Naturwissenschaften **70:** 200–201
19. **Masui H, Kondo T, Kojima M** (1989) An antifungal compound, 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid, from *Colocasia antiquorom* inoculated with *Ceratocystid fimbriata*. Phytochemistry **28:** 2613–2615
20. **Ohta H, Shida K, Peng YL, Furusawa I, Shishiyama J, Aibara S, Morita Y** (1990) The occurrence of lipid hydroperoxide-decomposing activities in rice and the relationship of such activities to the formation of antifungal substances. Plant Cell Physiol **31:** 1117–1122
21. **Patterson BD, MacRae EA, Ferguson IB** (1984) Estimation of hydrogen peroxide in plant extracts using titanium(IV). Anal Biochem **139:** 487–492
22. **Salaün JP, Weissbart D, Durst F, Pflieger P, Mioskowski C** (1989) Epoxidation of *cis* and *trans* Δ^9-unsaturated lauric acids by a cytochrome P-450-dependent system from higher plant microsomes. FEBS Lett **246:** 120–126
23. **Takahama U, Egashira T** (1991) Peroxidases in vacuoles of *Vicia faba* leaves. Phytochemistry **30:** 73–77