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Epoxidation of fatty acids, fatty methyl esters, and alkenes by immobilized oat seed peroxygenase☆

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Abstract

Fatty epoxides are used as plasticizers and plastic stabilizers and are intermediates for the production of other chemical substances. The currently used industrial procedure for fatty epoxide synthesis requires a strong acid catalyst which can cause oxirane ring opening and side product formation. To find a replacement for the acid catalyst, we have been conducting research on a peroxygenase enzyme from oat (*Avena sativa*) seeds and have devised a method for immobilization of this enzyme using a hydrophobic membrane support. In this study, fatty acids and fatty methyl esters commonly encountered in commercial vegetable oils were tested as substrates for immobilized peroxygenase, and the epoxide products were characterized. The epoxidation time course of linoleic acid showed two distinct phases with nearly complete conversion to monoepoxide before diepoxide was produced. The diepoxide formed from linolenic acid was found to be 9,10-15,16-diepoxy-12-octadecenoic acid, and only a trace of triepoxide was obtained. Additionally it was discovered that acyclic alkenes with internal double bonds, a cyclic alkene, and an alkene with an aromatic substituent were substrates of peroxygenase. However, alkenes with terminal unsaturation were unreactive. With every substrate examined, oat seed peroxygenase exhibited specificity for epoxidation, producing no other products, and oxirane ring opening did not occur.

Keywords: Atmospheric pressure chemical ionization mass spectrometry; *Avena sativa*; Coronaric acid; Electron impact mass spectrometry; Leukotoxin; Lipid epoxides; Oat seeds; Peroxygenase; Vernolic acid

1. Introduction

Epoxidation is an important industrial process; the annual production of ethylene oxide and propylene oxide in the US is estimated to be 2.9 and 1.2 million metric tonnes, respectively [1,2]. Long chain olefins, unsaturated fatty acids and esters, and various polymers are also subjected to epoxidation to give intermediates useful for the manufacture of commercially important materials. Each procedure requires a catalyst: production of ethylene oxide uses a silver catalyst; propylene oxide is produced using a homogeneous molybdenum catalyst or a heterogeneous titanium catalyst.

Fats and oils are renewable materials that can be chemically or enzymatically treated to produce materials that can often act as a replacement for materials derived from petroleum. Fatty epoxides are used directly as plasticizers and plastic stabilizers [3]. Due to the high reactivity of the oxirane ring, fatty epoxides are potentially useful intermediates for further

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elaboration. Currently fatty epoxides are prepared on an industrial scale from peracid formed in situ from hydrogen peroxide and acetic acid or formic acid using a strong mineral acid catalyst [4,5]. This procedure has low selectivity, and the acids must be recycled or treated before discharge into the environment. The low selectivity is mainly the result of partial hydrolysis to diols by trace levels of water under acidic conditions. The diols in turn act as nucleophiles for additional oxirane ring opening, resulting in the formation of higher molecular weight substances. The presence of these side products in commercial preparations of fatty epoxides diminishes their attractiveness as starting materials for further elaboration, as products may require expensive purification.

It would be desirable to replace the acidic peracid epoxidation procedure with one that uses an environmentally benign enzymatic catalyst which is functional near neutrality. Epoxidation promoted by cytochrome P450 and chloroperoxidase have received considerable attention due to their ability to promote asymmetric epoxidation [6–8]. Unfortunately chloroperoxidase is not active with larger substrates. Some types of cytochrome P450 catalysts are known to be active with fatty acids. However, epoxidation requires the use of a reductive cofactor which would limit commercial application. Several years ago an enzymatic activity termed peroxygenase was characterized in plant extracts by Blée [9], and Hamberg and Hamberg [10] while investigating the fate of lipid hydroperoxides. Peroxygenase, a hemoprotein, catalyzed the inter- and intramolecular transfer of oxygen from a fatty acid hydroperoxide to form epoxides on unsaturated fatty acids. It was found that an external oxidant, such as hydrogen peroxide or cumene hydroperoxide could be used as the source of oxygen [10,11]. Hamberg demonstrated that oat (*Avena sativa*) seeds are a good source of peroxygenase and examined the conversion of oleic acid to epoxide in the presence of hydrogen peroxide [12]. It was also found that linoleic acid was converted to epoxy, alcohol epoxy, and hydroxy fatty acids in a subcellular particle fraction. No oxidant was added, and product profiles were consistent with oxidation by the hydroperoxide generated by lipoxygenase acting on linoleic acid.

Since oat seeds are a relatively inexpensive source of peroxygenase, we have been investigating the feasibility of using peroxygenase for the preparation of fatty epoxides. We devised a method for the rapid isolation and immobilization of this peroxygenase on membranes [13] and conducting epoxidation reactions in organic solvent [14]. Larger scale epoxidations have been conducted using oleic acid as the substrate, and a high yield of stearic epoxide could be obtained with *t*-butyl hydroperoxide as the oxidant but not with hydrogen peroxide, as peroxygenase deactivation precluded high product formation [15]. The *trans* analogue of oleic acid, elaidic acid, was a poor substrate [13]. No other information on the substrate selectivity or specificity of immobilized oat seed peroxygenase is available, and this type of information is needed in order to form a basis for further experimentation on the enzymatic synthesis of epoxides from fats and oils. Here, we tested the activity of immobilized oat seed peroxygenase using *t*-butyl hydroperoxide as the oxidant on a variety of fatty acids and esters under conditions in which a majority of the faster reacting substrates were converted to oxidized products. Chosen for study were oleic, linoleic, and linolenic acids, major components of soybean and cotton seed oil, erucic acid, a major component of industrial rape seed oil, and ricinoleic acid, the major component of castor oil. We also demonstrated that peroxygenase is an active epoxide catalyst with some alkenes. All reaction products were examined using mass spectrometry linked to high performance liquid chromatography or gas chromatography, and we were able to demonstrate that the oat seed peroxygenase exhibits specificity for epoxide formation with no other products being detected.

2. Materials and methods

2.1. Chemicals

cis-2-Heptene, *trans*-2-heptene, *cis*-3-heptene, 1-heptene, 1-octene, 1-nonene, 2-methyl-1-heptene, cyclohexene, styrene, and methyl linolenate were from Aldrich. *t*-Butyl hydroperoxide (70%), oleic acid, methyl oleate, linoleic acid, methyl linoleate, linolenic acid, erucic acid, and ricinoleic acid were from Sigma.

2.2. Preparation of membrane-bound peroxygenase

Dry oat seeds (10 g) were ground in 5 g batches in a 37 ml Waring blender (New Hartford, CT) mini-jar

for 30 s. The ground oat seeds were transferred to a 110 ml mini-jar containing 90 ml of 0.1 M potassium phosphate buffer (pH 6.7) and blended for 90 s at high speed. The oat seed slurry was centrifuged at $9000 \times g$. The pellet was discarded, and the supernatant was centrifuged for an additional 10 min. The supernatant was divided into four equal portions, and each was vacuum-infiltrated through a Fluoropore membrane (0.2 μm, 47 mm) (Millipore, Bedford, MA). The Fluoropore membrane was wetted with methanol before loading onto the membrane holder.

2.3. Catalytic epoxidation

After vacuum infiltration, the Fluoropore membrane was washed with 20 ml CH_2Cl_2, cut into four equal pieces, and placed into a 50 ml stoppered Erlenmeyer flask. The flask contained reaction buffer containing 17.7 μmol substrate and 7.0 ml of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 0.1% (w/v) Tween 20, pH 7.5. The reactions were agitated at 20 °C for the indicated time. At the start of the reaction and again at 1, 2, and 4 h, 3.38 μmol *t*-butyl hydroperoxide was added. At 6 h, 20.26 μmol *t*-butyl hydroperoxide was added. At 24 h or as indicated, the reactions of fatty acids and methyl esters were terminated by adding 20 ml methanol and 10 ml water to each reaction. The pH was lowered to 3.0 with acid, and the reaction was extracted 2× with 50 ml portions of diethylether. The combined ether fractions were washed with water (2× 70 ml), dried over anhydrous sodium sulfate, and then ether was removed under a stream of nitrogen. Products were re-dissolved in 20 ml dichloromethane and methylated with diazomethane. Volatile hydrocarbons were sampled using solid phase microextractor fiber (100 μm) coated with polydimethylsiloxane (Supelco, Bellefonte, PA).

2.4. Chromatographic and instrumental methods

Proton and carbon nuclear magnetic resonance (NMR) spectrometry were obtained as described previously [14].

Epoxidized fatty methyl esters were analyzed by high performance liquid chromatography (HPLC). Solutions of the methyl ester were filtered with 13 mm, 0.45 μm syringe filters (PVDF, Scientific Resources, Eatontown, NJ). Dichloromethane was removed under a stream of nitrogen, and the products were dissolved in 1 ml isopropanol. Reaction mixtures were separated on two Symmetry 3.5 μm C_{18} reverse phase columns (150 mm × 2.1 mm and 50 mm × 2.1 mm) (Waters, Milford, MA) connected in series. Quantitation of epoxide formed was made with a Varex MK III evaporative light-scattering detector (Alltech, Deerfield, IL) operated at 55 °C, and with N_2 as the nebulizing gas at a flow rate of 1.5 l/min. Mobile phase composition and gradient was: 0–5 min H_2O/CH_3CN (40/60 (v/v)); 5–30 min H_2O/CH_3CN (40/60 (v/v)) to CH_3CN (100); 30–54 min CH_3CN (100). The flow rate was 0.25 ml/min. For epoxidized methyl erucate, the gradient time was lengthened 5 min. Products were further characterized by HPLC with mass detection using electron impact mass spectrometry (HPLC/EI-MS; Thermabeam mass detector, Waters, Milford, MA) and atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS; Micromass ZMD, Waters, Milford, MA). The EI-MS detector was set to scan in the mass range of *m*/*z* 55–600 at 1000 amu/s and had an ionization energy of 70 eV. Ionization source, nebulizer and expansion regions temperatures were 200, 64, and 75 °C, respectively. When using the APCI-MS, the HPLC gradient contained 0.1% formic acid, and the APCI-MS detector was set to scan in the mass range of *m*/*z* 150–550 at 400 amu/s. The corona, cone, and extractor voltages were 3700, 20, and 5 eV, respectively. The source and APCI heater temperatures were 150 and 400 °C, respectively.

Epoxidized aliphatic alkenes and styrene were sampled using a solid phase microextraction fiber, and the samples were injected directly onto a Hewlett-Packard (Wilmington, DE) 5890 Series II Plus gas chromatograph equipped with a HP 5972 mass selective detector set to scan from *m*/*z* 45–400 at 2 scans/s. A capillary column (Hewlett-Packard HP-5MS, 30 m × 0.25 mm) coated with 0.25 μm 5% PH ME siloxane was used to separate the products. The injector port temperature was 280 °C, and the detector transfer line temperature was 250 °C. The oven temperature was held at 35 °C for 5 min and was increased to 80 °C at 5 °C/min; increased to 240 °C at 35 °C/min and held at 240 °C for 5 min. Quantitation of product epoxide formation was made by following the total ion current.

2.5. Structural confirmation of epoxide products

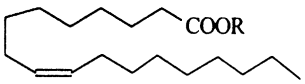
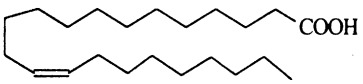
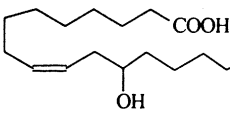
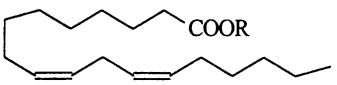
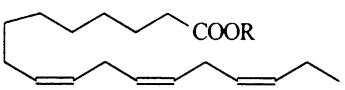
The number scheme is shown in Tables 1 and 2.

Methyl 9,10-epoxyoctadecanoate (*methyl stearate epoxide*; **1a**, **2a**). RT (mean retention time): 30.5 min. APCI-MS: *m/z* 354 [$M + 42$, addition of H^+ and CH_3CN], 313 [$M + 1$, addition of H^+], 295 [$M - 17$, addition of H^+, loss of H_2O], 263 [$M - 49$, addition of H^+, loss of H_2O and CH_3OH]. EI-MS: *m/z* 294 [$M - 18$, loss of H_2O], 281 [$M - 31$, loss of $^{\bullet}OCH_3$], 199 [$M - 113$, loss of $^{\bullet}(CH_2)_7CH_3$], 187 $[HO{=}CH(CH_2)_7COOCH_3]^+$, 155 [$M - 157$, loss of $^{\bullet}(CH_2)_7COOCH_3$] [18]. ^{1}H NMR (400 MHz, C_6D_6, δ_H): 1.13–1.17 (3H, t, $J = 7.0$, CH_3CH_2), 1.39–1.80 (26H, m, CH_2), 2.32–2.38 (2H, t, $J = 7.6$, CH_2COOCH_3), 2.97–3.02 (2H, bs, 9-*H* and 10-*H*), 3.61 (3H, s, $COOCH_3$). ^{13}C NMR (100 MHz, C_6D_6, δ_C): 15.24 (CH_3CH_2); 23.98, 26.12, 27.92, 28.04, 28.54, 29.28, 30.22, 30.43, 30.55, 30.84, 33.16, 34.99, 51.82 ($COOCH_3$); 57.55 (O–*C*H); 174.0 ($COOCH_3$) [16].

Methyl 13,14-epoxydocosenoate (*methyl behenate epoxide*; **3a**). RT: 43.5 min. APCI-MS: *m/z* 410 [$M + 42$], 369 [$M + 1$], 351 [$M - 17$], 337 [$M - 31$, addition of H^+, loss of CH_3OH], 319 [$M - 49$]. EI-MS: *m/z* 350 [$M - 18$], 337 [$M - 31$], 270 $[CH_3CH(O)CH(CH_2)_{11}COOCH_3]^{\bullet+}$, 255 [$M - 113$], 183 $[CH_3(CH_2)_{10}COOCH_3 - 31]^+$, 155 [$M - 213$, loss of $^{\bullet}(CH_2)_{11}COOCH_3$].

Methyl 9,10-epoxy-12-hydroxyoctadecanoate (*methyl hydroxystearate epoxide*; **4a**). RT: 12.16 min. APCI-MS: *m/z* 370 [$M + 42$], 329 [$M + 1$], 311

Table 1
Yields of fatty acid and ester epoxides in 24 h[a]

Substrate			Product	Yield (%)	Ratio
1	R = H		**1a**	99.8 ± 0.1	
2	R = CH_3		**2a**	93.9 ± 1.5	
3			**3a**	26.9 ± 2.2	
4			**4a**	73.6 ± 2.3	
5	R = H		**5a** (9,10-epoxy)	25.4 ± 6.3	1.09 ± 0.08 (**5a**/**5b**)
			5b (12,13-epoxy)	23.4 ± 5.7	
			5c (diepoxy)	46.7 ± 11.7	
6	R = CH_3		**6a** (9,10-epoxy)	17.6 ± 1.8	0.80 ± 0.05 (**6a**/**6b**)
			6b (12,13-epoxy)	21.9 ± 1.6	
			6c (diepoxy)	49.5 ± 5.2	
7	R = H		**7a** (monoepoxy)	0.6 ± 0.4	
			7b (9,10-15,16-diepoxy)	91.6 ± 2.2	
8	R = CH_3		**8a** (monoepoxy)	14.3 ± 2.3	
			8b (diepoxy)	78.3 ± 3.3	

[a] Assay conditions described in Fig. 1. Each result is the mean ± S.E. ($n = 3$–8).

Table 2
^{1}H and ^{13}C NMR of methyl 9,10-15,16-diepoxy-12-octadecenoate (**7b**, **8b**)

Chemical shift (400 MHz, C_6D_6, δ_H)	Number of protons	Appearance	Assignment	Coupling constant (J; Hz)
1.13–1.17	3H	t	C*H*$_3$CH$_2$	7.6
1.34–1.83	14H	m	CH$_2$	
2.23–2.40				
2.42–2.58	4H	m	11-*H*$_2$ and 14-*H*$_2$	
2.33–2.38	2H	t	CH$_2$COOCH$_3$	7.6
2.84–2.87				
2.92–2.98	2H	m	9-*H* and 16-*H*	
2.98–3.04	2H	m	10-*H* and 15-*H*	
3.61	3H	s	COOC*H*$_3$	
5.75–5.85	2H	bs	12-*H* and 13-*H*	

^{13}C NMR (100 MHz, C_6D_6, δ_C): 11.68 (*C*H$_3$CH$_2$); 22.37, 26.11, 27.74, 27.86, 28.61, 29.08, 30.19, 30.38, 30.49, 34.97, 51.86 (COO*C*H$_3$); 56.78, 56.90, 57.02, 57.53, 57.56, 58.66 (O–*C*H); 174.25 (*C*OOCH$_3$)

^{13}C NMR (100 MHz, $CDCl_3$, δ_C): 10.61 (*C*H$_3$CH$_2$); 21.09, 24.88, 26.38, 26.48, 26.55, 27.77, 29.02, 29.07, 29.17, 29.32, 34.05, 51.45 (COO*C*H$_3$); 56.21, 56.29, 56.34, 56.42, 57.12, 57.14, 58.31, 58.33 (O–*C*H); 126.8, 126.9 (*C*=*C*); 174.25(*C*OOCH$_3$)

[M − 17], 293 [M − 35, addition of H^+, loss of 2H_2O], 279 [M − 49]. EI-MS: m/z 310 [M − 18], 279 [M − 49, loss of $^{\bullet}OCH_3$ and H_2O], 243 [O=CH$_2$CH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$]$^{\bullet+}$, 225 [243 − 18], 187, 171 [M − 157], 155 [187 − CH$_3$OH].

Methyl 12,13-epoxy-9(*Z*)-octadecenoate (*methyl vernolate*, *methyl leukotoxin B*; **5a**, **6a**). RT: 27.18 min. APCI-MS: m/z 352 [M + 42], 311 [M + 1], 293 [M − 17], 279 [M − 31], 261 [M − 49]. EI-MS: m/z 292 [M − 18], 279 [M − 31], 207 [O≡CCH$_2$CH=CH(CH$_2$)$_7$COOCH$_3$ − 18]$^+$, 164 [M − 146, loss of $^{\bullet}$CH$_3$(CH$_2$)$_4$CH(O)CHCH$_3$ and 18], 147 [M − 163, loss of $^{\bullet}$CH$_3$(CH$_2$)$_4$CH(O)CH$_2$ and 31 and 18] [10,17].

Methyl 9,10-epoxy-12(*Z*)-octadecenoate (*methyl coronarate*; **5b**, **6b**). RT: 27.42 min. APCI-MS: m/z 352 [M + 42], 311 [M + 1], 293 [M − 17], 279 [M − 31], 261 [M − 49]. EI-MS: m/z 292 [M − 18], 279 [M − 31], 200 [CH$_2$(O)CH(CH$_2$)$_7$COOCH$_3$]$^{\bullet+}$, 168 [200 − 32], 155 [187 − CH$_3$OH], 125 [M − 185, loss of $^{\bullet}$CH$_3$(CH$_2$)$_4$CH=CHCH$_2$CH(O)CH$_2$ and 31], 109 [CH$_3$(CH$_2$)$_6$COOCH$_3$ − 49]$^+$ [10,17,18].

Methyl 9,10-12,13-diepoxyoctadecanoate (*methyl stearate diepoxide*; **5c**, **6c**). RT: 18.07 min. APCI-MS: m/z 368 [M + 42], 327 [M + 1], 309 [M − 17], 295 [M − 31], 277 [M − 49]. EI-MS: m/z 308 [M − 18], 295 [M − 31], 277 [M − 49], 211 [O=CHCH$_2$CH(O)CH(CH$_2$)$_7$CO]$^+$, 251 [CH$_2$CH(O)CHCH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 18]$^+$, 237 [CH$_2$CH(O)CHCH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 32]$^+$, 223 [O≡CCHCH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 31]$^{\bullet+}$, 211 [O=CHCH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 31]$^+$, 187, 165 [CH$_3$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 31 − 18]$^+$, 155 [187 − CH$_3$OH], 137 [O=CH(CH$_2$)$_7$COOCH$_3$ − 31 − 18]$^+$, 109 [CH$_3$(CH$_2$)$_6$COO*C*H$_3$ − 49]$^+$ [10,19]. ^{1}H NMR (400 MHz, C_6D_6, δ_H): 1.13–1.17 (3H, t, J = 7.6, C*H*$_3$CH$_2$), 1.34–1.82 (20H, m, C*H*$_2$), 1.82–1.87 (2H, t, J = 6.2,11-*H*$_2$), 2.32–2.38 (2H, t, J = 7.3, C*H*$_2$COOCH$_3$), 2.92–3.00 (2H, m, 9-*H* and 13-*H*), 3.14–3.21 (2H, m, 10-*H* and 12-*H*), 3.61 (3H, s, COOC*H*$_3$). ^{13}C NMR (100 MHz, C_6D_6, δ_C): 14.83 (*C*H$_3$CH$_2$); 23.57, 25.86, 27.21, 27.34, 27.58, 28.15, 28.44, 28.87, 29.00, 29.95, 30.13, 30.22, 32.62, 34.73; 51.57, 51.60 (COO*C*H$_3$); 54.53, 54.78, 54.81, 56.93, 56.95, 57.23 (O–*C*H); 174.0 (*C*OOCH$_3$) [16].

Methyl (mono)epoxyoctadecadienoate (**7a**, **8a**). RT: 23.28 min. APCI-MS: m/z 350 [M + 42], 309 [M + 1], 291 [M − 17], 277 [M − 31], 259 [M − 49]. EI-MS: m/z 236 [CH$_3$CH=CHCH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 18]$^{\bullet+}$, 207 [CH=CHCH$_2$CH(O)CH(CH$_2$)$_7$COOCH$_3$ − 32]$^+$, 175 [CH$_2$=CHCH$_2$CH=CH(CH$_2$)$_7$COOCH$_3$ − 49]$^+$, 155 [187 − 32], 147 [CH$_2$CH=CH(CH$_2$)$_7$COOCH$_3$ − 32 − 18]$^+$, 135 [CH$_2$=CH(CH$_2$)$_7$COOCH$_3$ − 49]$^+$, 121 [M − 187] [19]. ^{1}H NMR (400 MHz, C_6D_6, δ_H): 1.13–1.17 (3H, t, J = 7.5, C*H*$_3$CH$_2$), 1.34–1.83 (14H, m, C*H*$_2$), 2.20–2.30 (2H, m, 11-*H*$_2$ or 14-*H*$_2$), 2.32–2.38 (2H, t, J = 7.5, C*H*$_2$COOCH$_3$),

2.55–2.65 (2H, m, 14-H_2 or 11-H_2), 2.87–2.97 (1H, m, 9-*H* or 16-*H*), 3.00–3.10 (1H, m, 10-*H* or 15-*H*), 3.61 (3H, s, $COOCH_3$), 5.62–5.81 (4H, m, 12-*H*, 13-*H*, 15-*H*, 16-*H* or 9-*H*, 10-*H*, 12-*H*, 13-*H*). ^{13}C NMR (100 MHz, C_6D_6, δ_C): 11.40 (CH_3CH_2); 22.12, 25.89, 26.85, 27.35, 27.62, 28.22, 28.84, 30.04, 30.45, 34.74, 51.57 ($COOCH_3$); 56.79, 57.27, 58.39 (O–*C*H); 125.5, 131.4 (*C*=*C*); 174.2 ($COOCH_3$).

Methyl 9,10-15,16-diepoxy-12-octadecenoate (**7b**, **8b**). RT: 12.54 min. APCI-MS: *m*/*z* 366 [$M+42$], 325 [$M+1$], 307 [$M-17$], 293 [$M-31$], 275 [$M-49$]. EI-MS: *m*/*z* 221 $[CH_2CH(O)CHCH_2CH{=}CH(CH_2)_7COOCH_3-32]^+$, $[CH_2CH{=}CHCH_2CH(O)CH(CH_2)_7COOCH_3-32]^+$ or $[CH_3CH(O)CHCH_2CH(O)CH(CH_2)_7COOCH_3-49]^+$, 199 $[CH(O)CH(CH_2)_7COOCH_3]^+$, 167 [199 − CH_3OH], 155 [187 − CH_3OH], 137 [$M-187$], 135, 125 [$M-199$] [19]. For ^1H and ^{13}C NMR see Table 2.

2-Butyl-3-methyl-*cis*-oxirane (**9a**). RT: 8.78 min. GC–MS: *m*/*z* 113 [$M-1$], 99 [$M-15$], 85 [99–14], 71 [85–14].

2-Butyl-3-methyl-*trans*-oxirane (**10a**). RT: 7.72 min. GC–MS: *m*/*z* 113 [$M-1$], 99 [$M-15$], 85 [99 − 14], 71 [85 − 14].

2-Ethyl-3-propyl-*cis*-oxirane (**11a**). RT: 8.35 min. GC–MS: *m*/*z* 113 [$M-1$], 99 [$M-15$], 85 [99 − 14], 72 [85 − 13].

Pentyl oxirane (**12a**). RT: 9.72 min. GC–MS: *m*/*z* 85 [$M-(15+14)$], 71 [85 − 14].

7-Oxabicyclo-[4.1.0]-heptane (**16a**). RT: 7.68 min. GC–MS: *m*/*z* 97 [$M-1$], 83 [$M-15$], 69 [83 − 14], 55 [69 − 14].

Epoxyethyl benzene (**17a**). RT: 10.20 min. GC–MS: *m*/*z* 120 [*M*], 119 [$M-1$], 91 [119 − 28, loss of CO], 77 [91 − 14], 63 [77 − 14].

3. Results and discussion

3.1. Epoxidation of linoleic acid

Linoleic acid **5** was used as the substrate for epoxidation. Reactions were conducted for 2, 4, 6, and 24 h. The methylated reaction products were analyzed by HPLC, and the signal response from the evaporative light-scattering detector is plotted in Fig. 1 for each time. At the top (0 h), the methyl ester of substrate **5** eluted at 34.3 min. At 2 h, a doublet of peaks centered at 26.2 min arose as the peak from the methyl ester of **5** diminished. From analysis of the spectra from HPLC/APCI-MS and HPLC/EI-MS, the earlier peak of the doublet was assigned as the monoepoxy methyl ester **5b** (methyl 12,13-epoxy-9(*Z*)-octadecenoate). The later peak was found to be methylated monoepoxide **5a** (methyl 9,10-epoxy-11(*Z*)-octadecenoate). In the 4 h sample, substrate **5** was completely oxidized; only peaks corresponding to the monoepoxide isomers were present. In the 6 h sample, a new doublet of peaks arose at 17.1 min. Each peak of this doublet had an identical mass spectrum. Analysis by HPLC/APCI-MS and HPLC/EI-MS showed that these peaks were from diastereomers of the methyl ester of diepoxide **5c** (9,10-12,13-diepoxy-octadecanoate). In the 24 h sample, the peaks from diepoxide **5c** increased in size, and those from the monoepoxides **5a** and **5b** diminished. Several important conclusions can be drawn from these chromatograms: washing the membrane-bound peroxygenase preparation with CH_2Cl_2 prior to reaction removed endogenous lipid contaminants, particularly oleic acid and its epoxide. Allylic oxidation is not catalyzed by peroxygenase. The absence of polyhydroxy products shows that epoxide hydrolysis does not occur during the tested time span and, therefore, our peroxygenase preparation is not contaminated with epoxide hydrolase.

3.2. Epoxidation of fatty acids and esters

To determine the ability of peroxygenase to catalyze epoxide formation in different unsaturated substrates, individual aqueous reaction solutions of the tested substrates were prepared, and these were incubated with immobilized peroxygenase for 24 h. Epoxidation was initiated by the addition of *t*-butyl hydroperoxide, and this oxidant was added periodically over the course of the reaction as described in Section 2.3. The percentage yield of epoxide product was determined after work up of those reactions containing the fatty acids and fatty methyl esters or by sampling those reactions containing unsaturated hydrocarbons. Results for fatty acid and fatty methyl ester substrates are shown in Table 1. Compounds **1** and **2**, oleic acid and its methyl ester were almost completely converted into epoxide **1a** and **2a** during the 24 h reaction period. In contrast, only one quarter of erucic acid **3** and three quarters of ricinoleic acid **4** were converted to

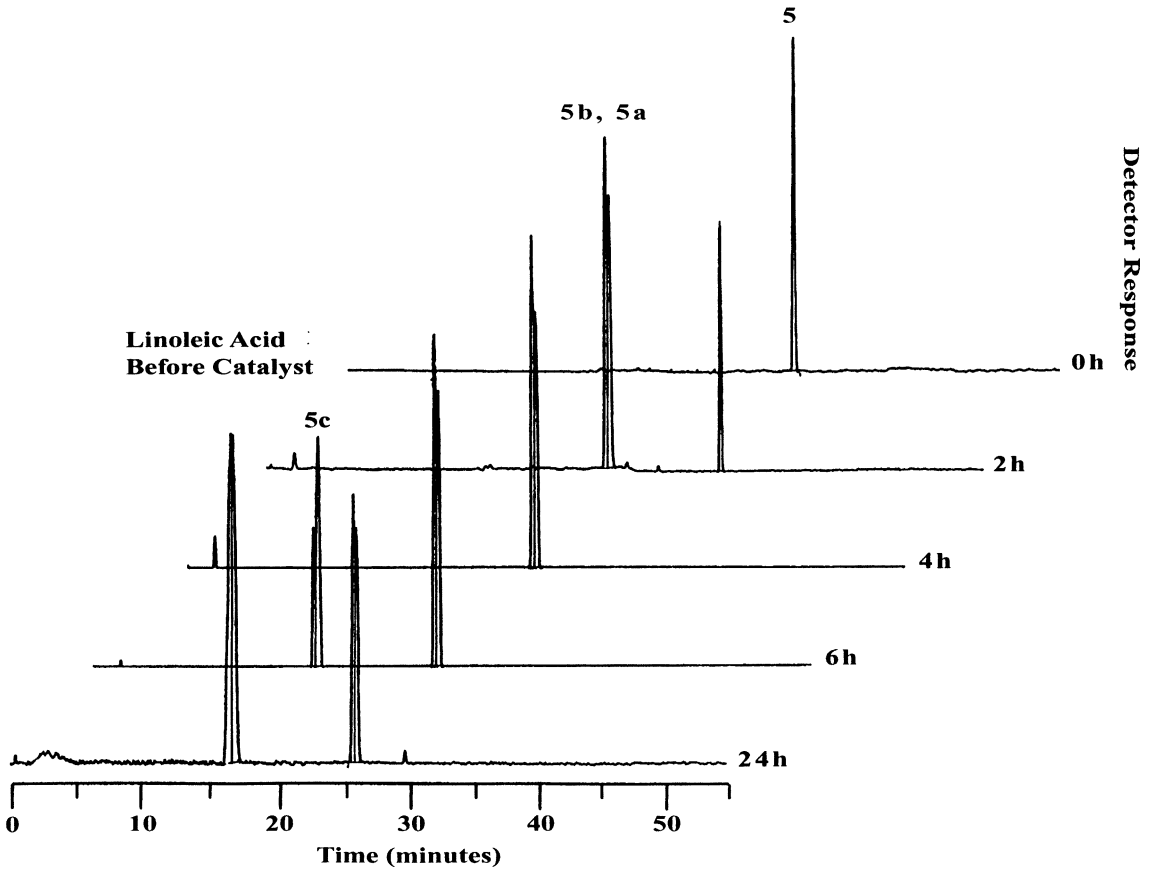


Fig. 1. Analysis of the epoxidation of linoleic acid **5** by reverse phase HPLC with an evaporative light-scattering detector. All materials were methylated before analysis. Each reaction contained 17.7 μmol substrate **5**, 7.0 ml 50 mM HEPES, 0.1% (w/v) Tween 20, pH 7.5, and peroxygenase immobilized on a Fluoropore membrane. At 0, 1, 2, and 4 h 3.38 μmol *t*-butyl hydroperoxide was added. At 6 h, 20.26 μmol *t*-butyl hydroperoxide was added. The reaction temperature was 20 °C. Retention times: methyl linoleate (methylated **5**), 34.3 min; monoepoxides (methylated **5a** and **5b**), 26.1–26.6 min; diepoxides (methylated **5c**), 16.9–17.3 min.

their respective epoxides (**3a** and **4a**) during this time period.

Linoleic acid **5** and its methyl ester **6** reacted in a nearly identical fashion, with all starting material epoxidized (Table 1 and Fig. 1). Epoxide product was nearly evenly divided between mono- and diepoxides. The ratio of mono 9,10-epoxy and 12,13-epoxy (**5a**/**5b**, **6a**/**6b**) was close to unity, demonstrating that the oat seed peroxygenase did not distinguish between the double bonds.

When linolenic acid **7** and methyl linolenate **8** were treated with peroxygenase, the reaction product consisted predominantly of diepoxides **7b** and **8b**, respectively; only a trace of triepoxides was detected. The HPLC method did not have sufficient resolving power to separate individual isomers of the monoepoxides **7a** and **8a**. The EI mass spectra of diepoxides **7b** and **8b** were insufficient to allow the assignment of regioisomers. Analysis of **7b** by ^{1}H and ^{13}C NMR showed unambiguously that only exterior double bonds were epoxidized (Table 2). This is shown mainly by the broad singlet and closely spaced doublets given by the double bond protons and carbons, respectively. Such behavior requires a similar electronic environment for the double bond protons and carbons. This situation is only satisfied by having

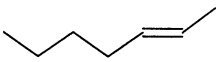
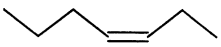
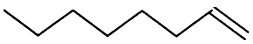
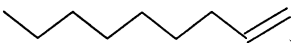
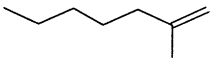
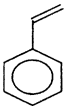
the double bond between each epoxide and, therefore, **7b** is methyl 9,10-15,16-diepoxy-12-octadecenoate.

The reactions with linolenic acid **7** were repeated with higher amounts of *t*-butyl hydroperoxide to determine whether triepoxide synthesis could be increased. A series of different concentrations of *t*-butyl hydroperoxide were used in an attempt to find the concentration that gave the highest triepoxide yields without deactivating the peroxygenase catalyst. However, even when the amount of *t*-butyl hydroperoxide was increased four-fold, most of the product was diepoxide, and only a trace of triepoxide could be observed.

3.3. Epoxidation of alkenes

In Table 3 are listed the results of epoxidations of nine unsaturated hydrocarbons. The highest yield of epoxide (**9a**, 93.9%) was obtained with *cis*-2-heptene (**9**). When the *trans* analogue **10** was used, the yield of epoxide **10a** decreased to 26.6%. Prior work has shown that oat seed peroxygenase acts slowly on elaidic acid, the *trans* analogue of oleic acid [13].

Table 3
Yields of alkene epoxides in 24 h[a]

Substrate		Product	Yield (%)
9		**9a**	93.9 ± 0.8
10		**10a**	26.6 ± 6.1
11		**11a**	72.7 ± 4.2
12		**12a**	2.8 ± 1.3
13		**13a**	0
14		**14a**	0
15		**15a**	15.4 ± 5.3
16		**16a**	70.5 ± 4.3
17		**17a**	57.4 ± 2.3

[a] Assay conditions described in Fig. 1.

cis-3-Heptene **11** was converted to epoxide **11a** in relatively high yield. The linear hydrocarbons **12**–**14** with terminal double bonds were either non-reactive (**13** and **14**) or showed very low reactivity (**12**). Substrate **15** with a methyl substitution on a terminal double bond gave a modest 15.4% yield of epoxide **15a**. Cyclohexene **16** and styrene **17** were converted to their epoxy derivatives relatively well and gave yields of epoxides **16a** and **17a** of 70.5 and 57.4%, respectively. Prior studies with chloroperoxidase, cytochrome P450, and hemins have shown that under oxidizing conditions, substances with terminal double bonds can act as alkylating agents, resulting in *N*-alkylated porphyrins [19]. This alkylation reaction is thought to be responsible for inactivation of chloroperoxidase. As noted above, however, substrates **15** and **17** gave rise to epoxides, though these also contain terminal double bonds. A similar pattern of reactivity was noted with chloroperoxidase in prior work [20]. It was suggested that compounds containing a terminal double bond with an adjacent substitution were unable to participate in *N*-alkylation due to the inability of these compounds to approach sufficiently close to the heme face to form a covalent bond.

3.4. Conclusions

Perhaps the most important finding is that oat seed peroxygenase exhibited specificity for epoxidation, a condition necessary to give minimal byproduct contamination. The absence of side products arising from oxirane ring opening is also beneficial in this regard. The finding that non-fatty acid alkenes are substrates for peroxygenase is important, and further research is needed with these substrates. Comparison of epoxide yields shows several types of selectivity by oat seed peroxygenase. Selectivity against formation of three epoxides in linolenic acid, methyl linolenate, and epoxidation of terminal double bonds in the alkenes was strong, and very little product was formed. The *trans* configuration double bond in the tested alkene was epoxidized slowly, as was elaidic acid in our previous work. Within the group of monounsaturated fatty acids or esters and *cis* alkenes that were tested, a more subtle type of discrimination was evident which depends on the position of the double bond and the structure of the substrate. Further studies in this area are warranted.

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References

[1] J.P. Dever, K.F. George, W.C. Hoffman, H. Soo, in: J.I. Kroschwitz, M. Howe-Grant (Eds.), Encyclopedia of Chemical Technology, vol. 9, 4th ed., Wiley, New York, 1994, p. 915.
[2] D.L. Trent, in: J.I. Kroschwitz, M. Howe-Grant (Eds.), Encyclopedia of Chemical Technology, vol. 20, 4th ed., Wiley, New York, 1994, p. 271.
[3] J.T. Lutz, in: M. Grayson (Ed.), Encyclopedia of Chemical Technology, vol. 9, Wiley, New York, 1980, p. 251.
[4] M. Wiebe, J. Kümmel, J. Prüß, H.-J. Warnecke, Lipids 100 (1998) 404.
[5] T.-C. Chou, J.-Y. Chang, Chem. Eng. Commun. 41 (1986) 253.
[6] F. van Rantwijk, R.A. Sheldon, Curr. Opin. Biotechnol. 11 (2000) 554.
[7] C.A. Martinez, J.D. Steward, Curr. Org. Chem. 4 (2000) 263.
[8] E.J. Allain, L.P. Hager, L. Deng, E.N. Jacobsen, J. Am. Chem. Soc. 115 (1993) 4415.
[9] E. Blée, in: G.J. Piazza (Ed.), Lipoxygenase and Lipoxygenase Pathway Enzymes, AOCS Press, Champaign, 1996, p. 138.
[10] M. Hamberg, G. Hamberg, Arch. Biochem. Biophys. 283 (1990) 409.
[11] E. Blée, F. Schuber, J. Biol. Chem. 265 (1990) 12887.
[12] M. Hamberg, G. Hamberg, Plant Physiol. 100 (1996) 807.
[13] G.J. Piazza, T.A. Foglia, A. Nuñez, Biotechnol. Lett. 22 (2000) 217.
[14] G.J. Piazza, T.A. Foglia, A. Nuñez, J. Am. Oil Chem. Soc. 76 (1999) 551.
[15] G.J. Piazza, T.A. Foglia, A. Nuñez, J. Am. Oil Chem. Soc. 78 (2001) 589.
[16] M.S.F. Lie Ken Jie, M.K. Pasha, Lipids 33 (1998) 633.
[17] T. Ozawa, M. Hayakawa, T. Takamura, S. Sugiyama, K. Suzuki, M. Iwata, F. Taki, T. Tomita, Biochem. Biophys. Res. Comm. 134 (1986) 1071.
[18] R. Kleiman, G.F. Spencer, J. Am. Oil Chem. Soc. 50 (1973) 31.
[19] A.F. Dexter, L.P. Hager, J. Am. Chem. Soc. 117 (1995) 817.
[20] A.F. Dexter, F.J. Lakner, R.A. Campbell, L.P. Hager, J. Am. Chem. Soc. 117 (1995) 6412.