One-Pot Synthesis of Fatty Acid Epoxides from Triacylglycerols Using Enzymes Present in Oat Seeds

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ABSTRACT: In our previous work we used ground oat (*Avena sativa*) seeds as an inexpensive source of enzymes for the modification of FA or their chemical derivatives. We have extended this work by observing the products derived from an intact fat and three vegetable oils through the concerted action of oat seed enzymes. A modified reversed-phase HPLC protocol was devised that allowed the quantification of FFA, epoxy FA, and acylglycerols without derivatization. It was found that the addition of the surfactant deoxycholate or calcium chloride was needed to observe best hydrolysis of TAG to FA. Without an added oxidant, lipase action produced up to 80% by weight of FA at pH 7–9 with small amounts of DAG and MAG. When the oxidant *t*-butyl hydroperoxide was added in the presence of deoxycholate, the FA were partially converted to epoxide derivatives (up to 35% by weight). When calcium chloride replaced deoxycholate, only 4% by weight of the product was epoxide derivatives. The highest levels of FA epoxides were generated at pH 7, and up to 15% by weight of the product at this pH was epoxymonostearin (monoepoxyoctadecanoylglycerol). The optimal molar amount of *t*-butyl hydroperoxide to add with deoxycholate for high epoxide formation was 1.4 to 2.8 times the amount of fatty esters in soybean oil. In no instance were oxidized DAG or TAG detected, demonstrating that a highly specific enzymatic process was responsible for epoxide formation. The epoxide products may be used to produce polyols with high viscosity for grease preparations.

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KEY WORDS: Avena sativa, fatty acid, fatty epoxide, hydroperoxide, lipase, oat seed, oxidation, peroxygenase.

Oat seeds contain a lipase enzyme that acts on a wide variety of acylglycerols and fatty esters to give FA as products. In aqueous media the lipase rapidly hydrolyzes fatty esters containing a double bond at $C_{9\text{-}10}$ (1). High lipolytic activity in aqueous media requires the presence of a surfactant to promote the formation of an emulsion or high levels of calcium ion (2). In a nonpolar organic solvent, the oat seed lipase shows little specificity and hydrolyzes a variety of oils and fats (3,4).

Epoxides of fats and oils and their FA derivatives are used as plasticizers and stabilizers of plastics. Since fatty epoxides can be converted to a number of chemical derivatives, there has been increasing interest in using these substances as intermediates in the production of new biobased industrial materials. As such, we have investigated the use of a peroxygenase enzyme found in oat seeds as a safe alternative to the currently used peracid epoxidation system (5–7). The aim of the current research was to observe the action of ground oat seeds on TAG to determine what epoxidized fatty materials can be obtained. Ground and defatted oat seeds were used as catalysts instead of purified enzymes to reduce labor inputs and ultimately the cost of epoxidized fatty materials. As shown in Scheme 1, there are available at least two possible pathways leading to epoxidized materials. In the first pathway (**A**) the acylglycerol **1** is converted to an epoxidized acylglycerol **2**. In the second pathway (**B**) acylglycerol **1** is hydrolyzed by lipase to glycerol and FA **3**–**5**. These are in turn epoxidized by peroxygenase to a number of derivatives shown by structures **6**–**10** (Scheme 1). We anticipated that very little oxidized acylglycerols would be formed since the oat seed lipase hydrolyzes the TAG substrates to FA rapidly. However, the activities of lipase and peroxygenase are dependent on the pH of the buffering medium, and it is shown in this work that at the optimal pH for peroxygenase activity, FA epoxides, and an epoxymonostearin (monoepoxyoctadecanoylglycerol) are the major oxidized products.

MATERIALS AND METHODS

Materials. Oat (*Avena sativa* L.) seeds were obtained from Davis Feed Mills (Perkasie, PA). Nu-Chek-Prep, Inc. (Elysian, MN) supplied triolein, trilinolein, and trilinolenin. Cumene hydroperoxide (80%), *t*-butyl hydroperoxide (TBHP; 70%), buffer components, and deoxycholate were purchased from Sigma (St. Louis, MO). Raw linseed oil was purchased from Sunnyside Corp. (Wheeling, IL). Soybean oil (SBO) and canola oil were purchased from a local supermarket. Water was purified to a resistance of 18 megohm-cm using a Barnstead E-pure system. All other reagents were of the highest purity available.

The oat seeds (5 g) were ground for 30 s in a blender cup (10 mL), defatted by shaking in diethyl ether (2 × 25 mL) for 10 min, and dried overnight in a vacuum desiccator. In a typical reaction procedure, vegetable oil or purified TAG (75 mg) was mixed with the ground, defatted oat seeds in 24 mL buffer containing

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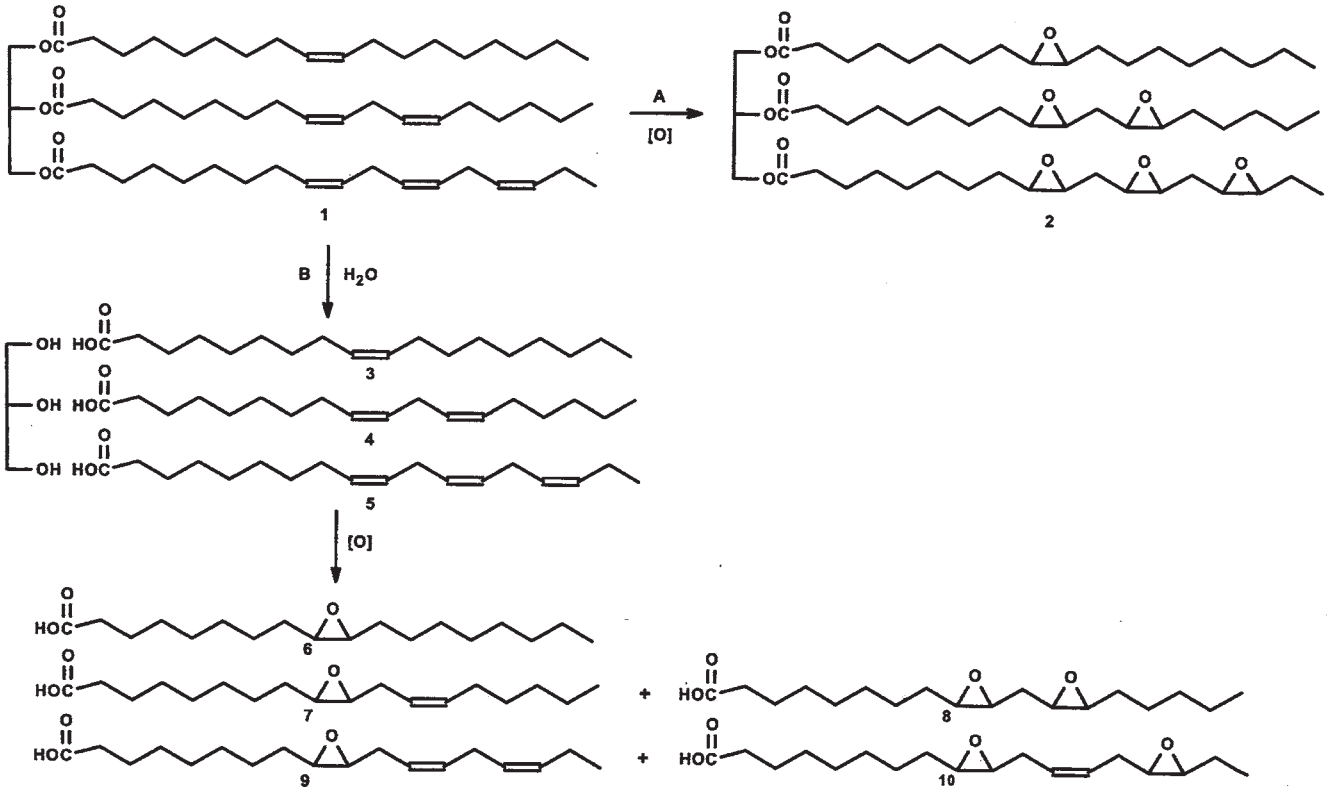

SCHEME 1

24 mg deoxycholate. The buffer consisted of four components, each with a different pK_a, to provide buffering capacity over a broad pH range to facilitate the determination of pH-activity profiles. Each buffer component was present at a concentration of 50 mM. The components were MES [2-(*N*-morpholino)ethanesulfonic acid], HEPES [*N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethansulfonic acid)], tricine [*N*-tris(hydroxymethyl)-methylglycine], and AMP [2-amino-2-methyl-1-propanol hydrochloride]. The mixture was shaken at 25°C for 24 h. For assays containing oxidatant 9.8 μL (71.6 μmol) of TBHP was added at 0, 1, 2, 4, and 6 h. At the end of the reaction, the mixture was acidified with 0.1 M HCl to pH 3.0, mixed with 30 mL diethylether, and vacuum filtered through 150 mm Whatman 113 filter paper. The filter was rinsed with an additional 30 mL diethyl ether, the filtrate was added to a separatory funnel, and the ether layer was removed. After washing the ether solution with 30 mL water, it was passed through a Millipore (Bedford, MA) 0.45 μm Durapore HVHP membrane filter and taken to dryness under a stream of nitrogen. The sample was dissolved in 1 mL isopropanol and refiltered on a 45 μm polyvinylidene difluoride (PVDF) syringe filter.

Separation and quantification of products and purification of epoxymonostearin. Hydrolysis products (FFA, DAG, and MAG) and epoxides were separated from unreacted TAG on Symmetry 3.5 μm C_{18} reversed-phase columns (150 × 2.1 mm and 50 × 2.1 mm) (Waters, Milford, MA). Products and unreacted TAG were detected with a Varex MK III ELSD (Alltech, Deerfield, IL) operated at 55°C, with N_2 as the nebulizing gas at a flow rate of 1.5 L/min. Mobile phase composition and gradient were 0–25 min H_2O/CH_3CN (30:70 vol/vol) to CH_3CN; 25–45 min CH_3CN to $(CH_3)_2CO$; 45–50 min $(CH_3)_2CO$ to CH_3CN; 50–55 min CH_3CN to H_2O/CH_3CN (30:70 vol/vol) at a flow rate of 0.25 mL/min. The solvent CH_3CN contained 0.05% acetic acid. Detector response was converted to mass using calibration curves prepared from SBO, oleic acid, and epoxystearic acid.

Epoxymonostearin (initially an unknown material) was separated from the other hydrolysis products, epoxides, and starting TAG by chromatography on two preparative HPLC columns using absorbance at 205 nm for detection. The first column was Adsorbosphere C18 (250 × 10 mm) (Alltech). Mobile phase composition and gradient used were 0–20 min $CH_3CN/(CH_3)_2CO$ (95:5 vol/vol); 20–35 min $CH_3CN/(CH_3)_2CO$ (95:5 vol/vol) to $(CH_3)_2CO$; 35–50 min $(CH_3)_2CO$; 50–55 min $(CH_3)_2CO$ to $CH_3CN/(CH_3)_2CO$ (95:5 vol/vol) at a flow rate of 2.0 mL/min. The material associated with an absorbance peak at 10–12 min was collected and then applied to a Dynamax Macro HPLC Si Column (300 × 10 mm; Rainin, Woburn, MA). Mobile phase composition and gradient used were 0–45 min C_6H_{14} to $CH_3CHOHCH_3/C_4H_8O/C_6H_{14}$ (25:25:50 by vol); 45–55 min $CH_3CHOHCH_3/C_4H_8O/C_6H_{14}$ (25:25:50 by vol) to C_6H_{14} at a flow rate of 2.0 mL/min. The material associated with an absorbance peak at 33–34 min was collected and found to be a pure substance by application to the Symmetry analytical HPLC column described above.

Product characterization. Starting TAG, FA, and epoxy derivatives were characterized by HPLC with mass detection using EI-MS (Thermabeam Mass Detector; Waters) and atmospheric pressure chemical ionization (APCI) HPLC-MS (Micromass ZMD; Waters) using the above HPLC conditions. The EI-MS detector was set to scan over the mass range of *m/z* 55–600 at a scan rate of 1000 *m/z* units/s and had an ionization energy of 70 eV. Ionization source, nebulizer, and expansion region temperatures were 200, 64, and 75°C, respectively. The APCI-MS detector was set to scan over the mass range of *m/z* 150–550 at a scan rate of 400 *m/z* units/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.

RESULTS AND DISCUSSION

Reaction pathway and quantification of products. Scheme 1 shows the reaction pathway of TAG to epoxy FA (pathway **B**), which is the major route to oxidized products expected with ground oat seeds because of the relatively high lipase activity found in oat seeds. The possibility that the peroxygenase acts directly on acylglycerols, however, to give epoxy products needs to be considered because there have been no prior investigations in this area with peroxygenase from oat seeds. What is needed is an HPLC method that separates FA, acylglycerols and their oxidized products. Accordingly, the HPLC method given in the Materials and Methods section was developed. The gradient procedure is a modification of a procedure previously used to separate fatty methyl esters, amides, and their epoxy products (5,8). Key changes were the addition of 0.05% acetic acid to CH_3CN, and the addition of an acetone step to elute the DAG and TAG. The fatty epoxides produced here are either isolated or separated from adjacent double bonds by a methylene group. As such they are relatively stable toward acid hydrolysis. Examples of elution profiles obtained with this gradient after reaction with three oils and a fat are discussed below.

Influence of deoxycholate on glyceride hydrolysis and epoxidation. Our attempts to conduct hydrolysis/epoxidation in aqueous buffer with no surfactant gave poor results. That very limited hydrolysis/epoxidation occurred was consistent with our earlier results that showed hydrolysis by oat seed lipase occurs rapidly only in the presence of an emulsifier or high calcium ion concentration (2). We decided to concentrate our efforts on the use of the surfactant deoxycholate because our results with glyceride oxidation with lipoxygenase showed this surfactant to be compatible with high enzyme activity (9). Deoxycholate also does not interfere with subsequent product analysis. Studies were conducted at pH 7.5 with TBHP, SBO, and varying amounts of the surfactant deoxycholate present in the aqueous buffer medium. The rate of conversion of SBO increased as the amount of deoxycholate was increased up to 0.1% (wt/vol). Further increases in deoxycholate did not provide better conversion of SBO to FA or FA epoxides; therefore, subsequent experiments were performed with 0.1% deoxycholate present in the buffered media.

Influence of pH on FA and epoxide formation. Incubations of ground oat seeds with SBO in the presence of aqueous buffer containing 0.1% deoxycholate were conducted at different pH values. In these experiments no TBHP oxidant was added. Figure 1 shows the averages obtained from several pH trials. It can be seen that the plot of liberated FA vs. pH is approximately bell-shaped. The maximal amount of FA is released in the range of pH 7–9. At pH values above and below this range, the amount of released FA decreases. Approximately 80% by weight of the product was FA when the reactions were conducted at pH 7–9. When 60 mM calcium chloride was added to replace deoxycholate, the plot of liberated FA vs. pH was also bell-shaped, and at pH 9 approximately 85% of the product weight was FA.

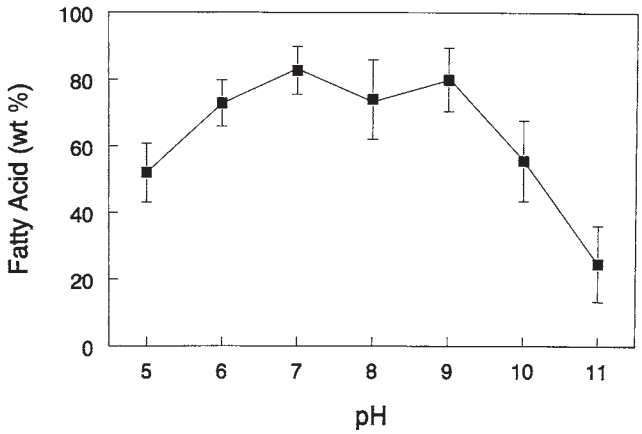

FIG. 1. The effect of aqueous buffer pH on FA released from soybean oil (SBO) by lipase in ground oat seeds. SBO (75 mg) was added to ground, defatted oat seeds (5 g) in 24 mL aqueous buffer at the indicated pH containing 0.024 g deoxycholate. The composition of the buffer is given in the Materials and Methods section. The mixture was shaken at 25°C for 24 h. Data are the mean ± SE for $n = 4$.

Incubations of ground oat seeds with SBO were repeated in the presence of 0.1% deoxycholate under conditions identical to those above except that the oxidant TBHP was added in repeated aliquots over a 6-h period. The results are displayed in Figure 2, which shows a plot of the weight percentage of the products obtained after incubation for 24 h. As will be discussed more fully shortly, these species constitute most of the observed hydrolysis products. Not shown Figure 2 are DAG and a very small amount of polar material near the beginning of the chromatogram whose chemical structure was not determined. The amount of TAG remaining was minimal at pH 7 and was higher at pH values both lower and higher than pH 7. Conversely, the amount of FA was greatest in the pH range of 7–9. Somewhat surprisingly, the highest level of epoxy FA was observed at pH values of 6 and 7, indicating that peroxygenase is more active at lower pH values than lipase. That no epoxidized DAG and TAG were detected indicates that these species are not good substrates for the oat peroxygenase enzyme. The lack of epoxidized DAG and TAG also indicates that epoxide formation by lipase-created peracid is not operative here, since peracid epoxidation would not show discrimination between the double bonds in FA and those in DAG and TAG. In analyzing the products, an unfamiliar peak was noted that eluted just prior to the diepoxide derivative from linoleic acid and constituted as much as 15% of the mass of the products when reactions were conducted at pH 7. A high M.W.

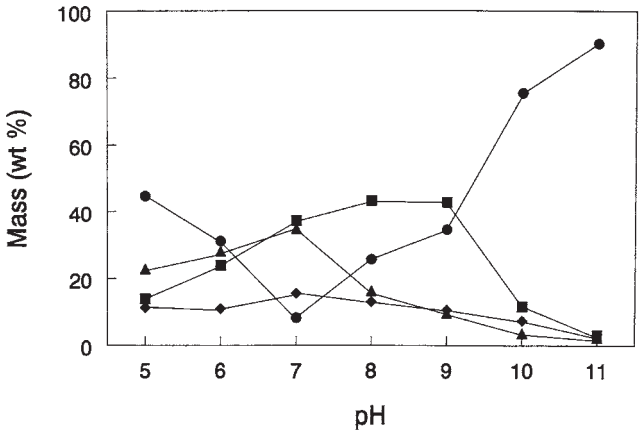

FIG. 2. The effect of aqueous buffer pH on TAG, FA, FA epoxide, and MAG epoxide generated from SBO by the concerted action of lipase and peroxygenase in ground oat seeds. SBO (75 mg) was added to ground, defatted oat seeds (5 g) in 24 mL aqueous buffer at the indicated pH containing 0.024 g deoxycholate. The composition of the buffer is given in the Materials and Methods section. The mixture was shaken at 25°C for 24 h. At 0, 1, 2, 4, and 6 h TBHP (71.6 ìmol *t*-butyl hydroperoxide, 9.8 µL) was added. Data are the mean for $n = 3$. Error bars are not shown to clarify the graph. Figure legends: (■) FA, (▲) epoxy FA, (●) TAG, (◆) epoxymonostearin. For other abbreviation see Figure 1.

as indicated by MS and relatively high polarity suggested that the material was an oxidized acylglycerol. However, the M.W. of acylglycerols are often difficult to determine with certainty by commonly available MS techniques owing to the rapid loss of water resulting in low-intensity peaks for M or M+1. Consequently the unknown material was purified by preparative reversed-phase and normal-phase HPLC as described in the Materials and Methods section, silylated, and subjected to analysis by positive-ion APCI-MS. A base peak at $m/z = 517$ was observed. This corresponds to the M.W. of epoxymonostearin (monoepoxyoctadecanoylglycerol) containing two trimethylsilyl groups and a proton.

When deoxycholate was replaced with calcium chloride, the rate of oxidation to epoxy FA decreased so that at most 4% by weight of the product was epoxy FA at pH 7, and less than this at other pH values. At all pH values examined, less than 1% of the product weight was epoxymonostearin . Thus, whereas lipase activity is stimulated by calcium chloride, peroxygenase activity is not stimulated; addition of deoxycholate is necessary to observe high epoxide formation.

Figure 3 shows the analysis by HPLC with ELSD detection of the hydrolysis and oxidation of canola oil, linseed oil, SBO, and tallow by the lipase and peroxygenase present in ground

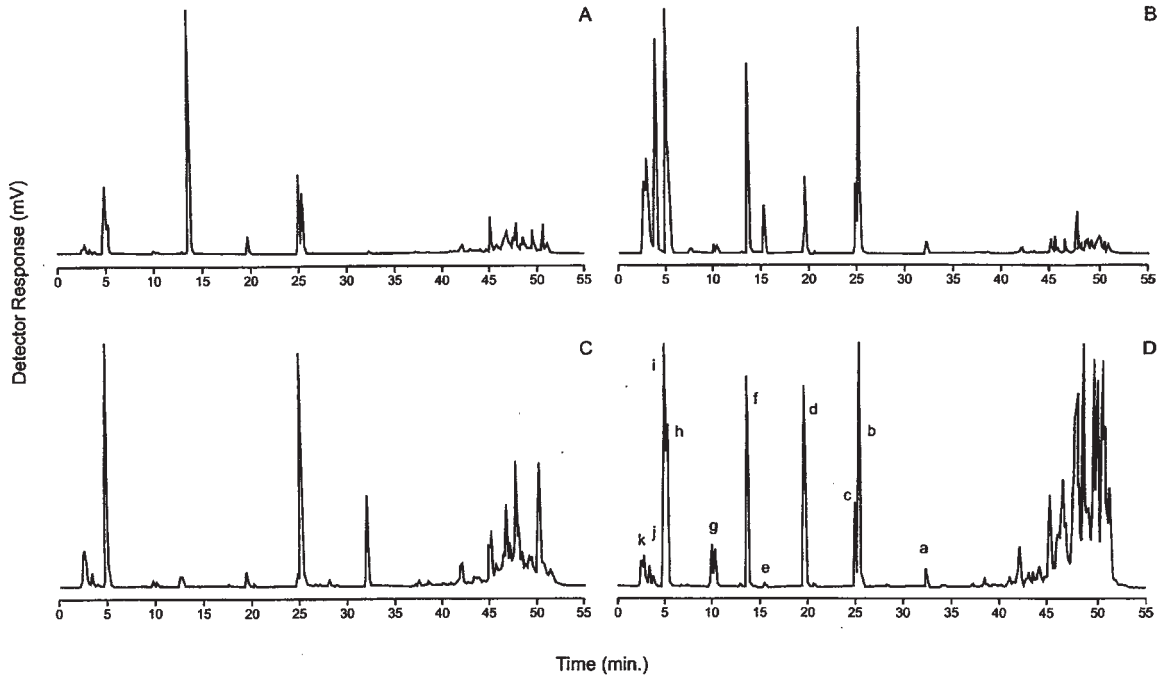

FIG. 3. Analyses of hydrolyzed and epoxidized products from the action of oat seed lipase on canola oil (**A**), linseed oil (**B**), tallow (**C**), and SBO (**D**). The oils and tallow (75 mg) were hydrolyzed and epoxidized at pH 6 in the presence of deoxycholate and TBHP as described in the legend for Figure 2. The figures show the signal trace of an ELSD detector from HPLC analyses that were performed as described in the Materials and Methods section. Composition of labeled peaks: **a**, stearic acid (octadecanoic acid); **b**, oleic acid [9(*Z*)-octadecenoic acid]; **c**, palmitoleic acid [9(*Z*)-hexadecenoic acid]; **d**, linoleic acid [9(*Z*),12(*Z*)-octadecadienoic acid]; **e**, linolenic acid [9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid]; **f**, monoepoxide derivative from oleic acid (9,10-epoxyoctadecanoic acid); **g**, monoepoxide derivatives from linoleic acid [9,10-epoxy-12(*Z*)-octadecenoic acid and 12,13-epoxy-9(*Z*)-octadecenoic acid]; **h**, mixture of diepoxide derivative from linoleic acid and monoepoxide derivatives from linolenic acid [9,10-12,13-diepoxy-octadecanoic acid, 9,10-epoxy-12(*Z*),15(*Z*)-octadecadienoic acid, and 15,16-epoxy-9(*Z*),12(*Z*)-octadecadienoic acid]; **i**, epoxymonostearin (monoepoxyoctadecanoylglycerol); **j**, diepoxide derivative from linolenic acid [9,10-15,16-diepoxy-12(*Z*)-octadecenoic acid]; **k**, triepoxide derivative from linolenic acid (9,10-12,13-15,16-octadecanoic acid). The unmarked area from 41 min and longer contained TAG. The area at times under 41 min contained DAG. DAG could be detected down to 27 min using atmospheric pressure chemical ionization (APCI)-MS, but as seen from the ELSD traces, there is very little mass associated with the DAG fraction. For abbreviations see Figures 1 and 2.

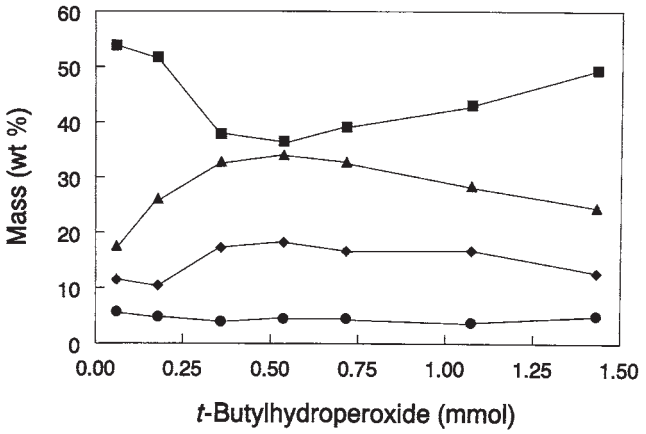

FIG. 4. The effect of amount of TBHP added on TAG, FA, FA epoxide, and epoxymonostearin generated from SBO by the concerted action of lipase and peroxygenase in ground oat seeds. Assays were conducted for 24 h as described for Figure 2 except different amounts of TBHP were added, and the pH was 7.0. The TBHP was divided into five separate portions added at 0, 1, 2, 4, and 6 h. The TBHP amount shown on the figure is the total amount added. Note that 0.358 mmol TBHP (third data point from the left) is equivalent to that used in Figures 2 and 3. The data are the average of two repetitions. Figure legends:(■) FA, (▲) epoxy FA, (●) TAG, (◆) epoxymonostearin. For abbreviations see Figures 1 and 2.

oat seeds. All reactions contained TBHP and deoxycholate. The peaks on SBO (trace D) have been labeled since the HPLC-MS work was completed initially with this oil, but the assignments have been confirmed by MS analysis with the other oils and tallow. The trace from SBO shows high levels of oleic, linoleic, and linolenic acids, as well as fairly high levels of the monoepoxide from oleic acid (9,10-epoxyoctadecanoic acid), the monoepoxide from linolenic acid [9,10-epoxy-12(*Z*),15(*Z*)-octadecadienoic acid, and 15,16-epoxy-9(*Z*),12(*Z*)-octadecadienoic acid], the diepoxide from linoleic acid (9,10-12,13-diepoxyoctadecanoic acid), and epoxymonostearin. The major product given by canola oil (trace A) is the epoxystearic acid (9,10-epoxyoctadecanoic acid), reflecting the high proportion of oleate in this oil. In addition to epoxystearic acid, the major products given by linseed oil (trace B) are epoxymonostearin and the monounsaturated diepoxide from linolenic acid [9,10-15,16-diepoxy-12(*Z*)-octadecenoic acid]. A peak due to triepoxystearic acid (9,10-12,13-15,16-octadecanoic acid) is also prominent in the ELSD trace. The products from tallow (trace C) are unique because very little epoxy FA is evident. The major oxidized product is the epoxymonostearin. There is also a large peak for oleic acid.

Influence of the oxidant TBHP on epoxide formation. The total amount of TBHP was varied by 25-fold to determine whether increasing TBHP changed the amounts of epoxy FA and epoxymonostearin generated. In the prior experiments 9.8 μL (71.6 μmol) TBHP was added at five separate time points (0, 1, 2, 4, 6 h) for a total of 0.358 mmol of TBHP. Additional experiments were conducted at pH 7.0 with the amount of TBHP ranging between 0.060 and 1.43 mmol. The results are shown in Figure 4. Here one can see that there was little change in the amount of FA, epoxy FA, and epoxymonostearin as TBHP was increased from 0.060 to 0.358 mmol. Increasing the amount of TBHP beyond this point caused a decrease in the levels of the epoxy materials produced, presumably due to overoxidation of peroxygenase by TBHP. We had observed this behavior before using more highly purified fractions of peroxygenase (10).

Thus, we were able to obtain up to 35% epoxy FA from SBO by using only ground oat seeds as the source of enzymes in the presence of deoxycholate. The epoxymonostearin generated in these reactions has not been observed previously from preparations of oat seeds. Preparations containing epoxy FA and epoxymonostearin can be easily hydrolyzed to polyol FA, and lithium salts of the polyol FA can be prepared to form a component of a high-temperature grease.

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