Optimizing Reaction Parameters for the Enzymatic Synthesis of Epoxidized Oleic Acid with Oat Seed Peroxygenase

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ABSTRACT: Peroxygenase is a plant enzyme that catalyzes the oxidation of a double bond to an epoxide in a stereospecific and enantiofacially selective manner. A microsomal fraction containing peroxygenase was prepared from oat (*Avena sativa*) seeds and the enzyme immobilized onto a hydrophobic membrane. The enzymatic activity of the immobilized preparation was assayed in 1 h by measuring epoxidation of sodium oleate (5 mg) in buffer-surfactant mixtures. The pH optimum of the reaction was 7.5 when *t*-butyl hydroperoxide was the oxidant and 5.5 when hydrogen peroxide was the oxidant. With *t*-butyl hydroperoxide as oxidant the immobilized enzyme showed increasing activity to 65°C. The temperature profile with hydrogen peroxide was flatter, although activity was also retained to 65°C. In 1 h reactions at 25°C at their respective optimal pH values, *t*-butyl hydroperoxide and hydrogen peroxide promoted epoxide formation at the same rate. Larger-scale reactions were conducted using a 20-fold increase in sodium oleate (to 100 mg). Reaction time was lengthened to 24 h. At optimized levels of *t*-butyl hydroperoxide 80% conversion to epoxide was achieved. With hydrogen peroxide only a 33% yield of epoxide was obtained, which indicates that hydrogen peroxide may deactivate peroxygenase.

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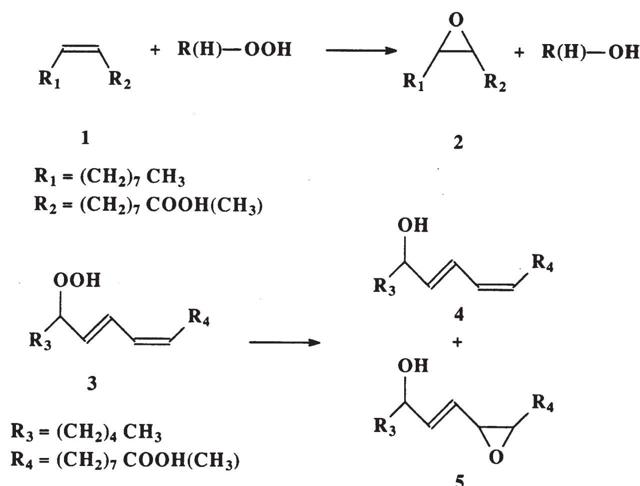
KEY WORDS: *Avena sativa*, *t*-butyl hydroperoxide, epoxide, hydrogen peroxide, peroxygenase, sodium oleate.

Soybean oil and the esters of tall oil fatty acids are converted to their epoxy derivatives for commercial applications. The primary market for these materials is as plasticizers for polyvinyl chloride (PVC) resins (1). Recent proposals to ban phthalate plasticizers have sparked renewed interest in epoxidized oils and esters for use in soft PVC toys (2). Additionally there is interest in the use of epoxidized oils in the production of paints and coatings with low levels of volatile organic compounds (VOC), as mandated by recently enacted antipollution legislation and regulations (3).

Epoxidized oils and fatty acids are produced on an industrial scale using peracids (4). The peracids are generated *in situ* by reacting formic or acetic acid with hydrogen peroxide in the presence of a strong acid catalyst. A disadvantage of this procedure is that the strong acid catalyzes oxirane ring opening, causes equipment corrosion, and must be recycled or neutralized before discharge to the environment. Also the peracid intermediate is unstable, and explosive conditions are possible.

The epoxide functionality serves as a valuable chemical intermediate, transformable to a wide variety of products. Thus there is a continuing search for better methods for promoting epoxidation (5). One method that we have recently investigated uses the enzyme peroxygenase as a catalyst. This enzyme promotes the stereospecific and enantiofacially selective transfer of an oxygen atom from an organic hydroperoxide or hydrogen peroxide to a double bond receptor (6,7).

Scheme 1 illustrates two reactions catalyzed by peroxygenase: formation of epoxide **2** with externally added hydroperoxide (top) and peroxygenase cleavage of fatty acid hydroperoxide **3** with and without inter- or intramolecular oxygen transfer to form alcohol **4** and epoxy alcohol **5** (bottom). We were able to demonstrate that the peroxygenase present in oat (*Avena sativa*) seeds catalyzes the conversion of fatty acid hydroperoxide **3** to the corresponding alcohol **4** and epoxy alcohol **5** in a variety of nonpolar organic solvents, as well as aqueous media (8). Subsequent studies showed that peroxygenase could be bound to hydrophobic membranes and used



SCHEME 1

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in both aqueous and organic solvents to convert oleic acid **1** to its corresponding epoxide **2** with one of several organic hydroperoxides or hydrogen peroxide (9). Blée and Schuber (10) have presented evidence that indicates that peroxygenase provides catalysis through the action of a heme-iron-activated oxidative species in an active site pocket. Our prior results are in accord with this view (9). In particular, epoxidation is accomplished cleanly with minimal by-product formation, and the *trans* double bond elaidic acid does not react rapidly; these observations are inconsistent with a free radical mechanism. In this study, the pH and temperature dependence of epoxidation by membrane-bound peroxygenase was examined to determine optimal reaction conditions. Studies on larger-scale reactions also were conducted as a necessary prerequisite for synthesizing sufficient quantities of epoxide for product testing.

MATERIALS AND METHODS

Materials. Oat seeds (*A. sativa* L.) were supplied by Equine Speciality Feed Co. (Ada, MN). Durapore (PVDF, hydrophilic) membranes and Fluoropore (PTFE, hydrophobic) membranes were from Millipore (Bedford, MA). Sodium oleate was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Heptane and hydrogen peroxide (30%) were purchased from Aldrich (Milwaukee, WI). Sigma (St. Louis, MO) was the source of *t*-butyl hydroperoxide (70%). Water was purified to a resistance of 18 mΩ-cm using a Barnstead (Dubuque, IA) NANO pure system. All other reagents were of the highest purity available.

Preparation of oat seed microsomes. For small-scale reactions, dry oat seeds (10 g) were ground in 5-g batches in a 37-mL Waring Blender (New Hartfod, CT) mini-jar for 30 s. The ground oat seeds were transferred to a 110-mL mini-jar containing 90 mL cold 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 × *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged for an additional 10 min at 9000 × *g*. After the second centrifugation, the supernatant was divided into four equal portions. Each portion was subjected to vacuum infiltration with a Fluoropore membrane (0.2 µm, 47 mm). The Fluoropore membrane was wetted with methanol before loading onto the membrane holder. After vacuum infiltration, the membrane was cut into four equal size pieces, and these pieces were placed into a reaction flask.

Fluoropore membranes from two types of treatments were used to test for nonenzymatic oxidation. The Fluoropore membrane was wetted with methanol, and then 35 mL of 0.1 M potassium phosphate buffer (pH 6.7) was passed through the membrane using vacuum infiltration. The Fluoropore membrane was loaded with the oat seed peroxygenase preparation, and then the loaded membrane was submersed in boiling water for 30 s.

For large-scale reactions 100 g of oats were ground dry, and then the ground seeds were homogenized in 900 mL of cold 0.1 M potassium phosphate buffer (pH 6.7) using a Waring commercial blender. The slurry was centrifuged at 16,000 × *g* for 15 min. The pellet was discarded, and the supernatant was centrifuged for an additional 15 min at 16,000 × *g*. The supernatant was passed through a hydrophilic Durapore membrane filter (0.65 µm, 142 mm), and the filtrate was collected. This was divided into quarters and each was passed through a hydrophobic Fluoropore membrane (0.2 µm, 142 mm).

Epoxidations. Smaller-scale reactions, used for determining pH and temperature profiles, contained 5 mg (16.4 µmol) sodium oleate, 6.3 mL buffer, 0.7 mL 1% (wt/vol) Tween 20, and 7.3 µmol *t*-butyl hydroperoxide or H_2O_2. The buffer consisted of four components, each with a different pK_a, to provide buffering capacity over a broad pH range. Each component was present at a concentration of 50 mM. The components were tricine [*N*-tris(hydroxymethyl)-methylglycine], MES [2-(*N*-morpholino)ethanesulfonic acid], HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)], and glacial acetic acid. Each assay was agitated at 20°C for 1 h or as indicated. Larger-scale reactions contained 100 mg (0.329 mmol) sodium oleate and 32.4 mL buffer. The buffer consisted of 50 mM HEPES/0.1% (wt/vol) Tween 20, pH 7.5, for reactions using *t*-butyl hydroperoxide or 50 mM MES/50 mM glacial acetic acid/0.1% (wt/vol) Tween 20, pH 5.5, for reactions using hydrogen peroxide. Products were extracted and analyzed by high-performance liquid chromatography as described earlier (9).

RESULTS AND DISCUSSION

Optimizing reaction parameters. Before proceeding to larger-scale reactions, it was necessary to determine reaction conditions that promoted the most rapid rate of epoxide formation. To accomplish this, reaction time was restricted to 1 h and suboptimal (less than stoichiometric) quantities of oxidant were added, as prior work had indicated that higher oxidant levels deactivate peroxygenase (11–13). Figure 1 shows the effect of buffer pH on the yield of epoxide formed from sodium oleate in 1 h. The highest yield of epoxide with *t*-butyl hydroperoxide was obtained at pH 7.5–8.0. Hydrogen peroxide gave the highest yield of epoxide at pH 5.5. Note, however, that the yield of epoxide was approximately identical for both *t*-butyl hydroperoxide and hydrogen peroxide at their pH optima. Support for this conclusion was obtained by concurrent 1-h assays of sodium oleate epoxidation with *t*-butyl hydroperoxide at pH 7.5 and with hydrogen peroxide at pH 5.5. The results of four repetitions with each oxidant were *t*-butyl hydroperoxide: 32.4 ± 0.9% and hydrogen peroxide: 29.1 ± 2.4%.

Figure 2 shows the influence of temperature on the yield in 1-h reactions. When the oxidant *t*-butyl hydroperoxide was used, the yield of epoxide increased as the temperature was increased to 65°C and then decreased slightly at 75°C. The yield of epoxide at higher temperatures was the maximum possible given the amount of *t*-butyl hydroperoxide added. Control experiments were performed using Fluoropore mem-

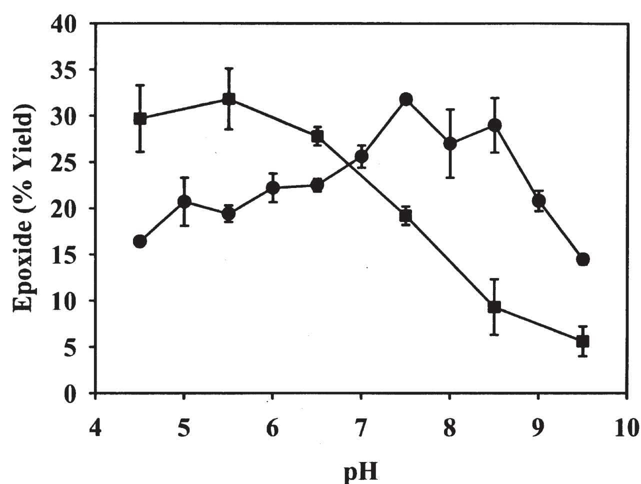

FIG. 1. Effect of pH on the epoxidation of sodium oleate by peroxygenase supported on a Fluoropore membrane. Assays contained 5 mg (16.4 µmol) sodium oleate, 6.3 mL buffer, 0.7 mL 1% (wt/vol) Tween 20, and 7.3 µmol *t*-butyl hydroperoxide (●) or H_2O_2 (■). Assays were conducted for 1 h at 20°C. Percentage yield calculations based on sodium oleate (theoretical maximum 44%). Each data point is the mean ± SE (*n* = 2–6).

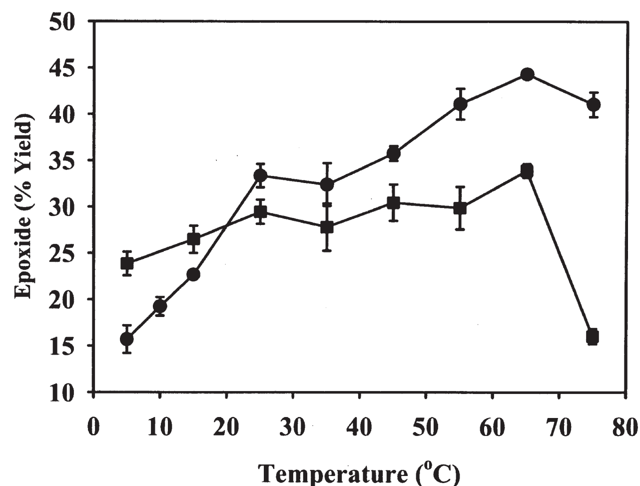

FIG. 2. Effect of temperature on the epoxidation of sodium oleate by peroxygenase supported on a Fluoropore membrane. Assays were conducted at pH 7.5 for *t*-butyl hydroperoxide (●) and pH 5.5 for H_2O_2 (■). Other assay conditions as in Figure 1.

branes treated only with methanol and buffer as described in the Materials and Methods section. Additional control experiments were performed with Fluoropore membranes containing peroxygenase that were immersed in boiling water for 30 s. Reactions with sodium oleate, *t*-butyl hydroperoxide, and these membranes under conditions described in Figure 1, except at 65°C, gave no detectable epoxide formation. The influence of temperature when hydrogen peroxide was the oxidant is also shown in Figure 2. Increases in temperature resulted in only modest increases in the epoxide yield, and at 75°C the epoxide yield decreased. Note, however, that at 25°C, the yield of epoxide was approximately the same for both *t*-butyl hydroperoxide and hydrogen peroxide.

Larger-scale reactions. Oat seeds and sodium oleate were

TABLE 1
Larger-Scale Production of Epoxide with *t*-Butyl Hydroperoxide (*t*-BuOOH) or Hydrogen Peroxide

Time (h)	*t*-Butyl hydroperoxide[a] or H_2O_2[b] added (µmol)			
	Reaction A	Reaction B	Reaction C	Reaction D
0	270	135	67.5	33.8
1	270	135	67.5	33.8
2	270	135	67.5	33.8
4	270	135	67.5	33.8
6	810	405	203	101
Total	1890	946	473	236
	Epoxide yield at 24 h[c] (µmol)			
t-BuOOH	191	258	262	230
H_2O_2	33.9	42.0	74.9	108
	Percentage yield based on sodium oleate			
t-BuOOH	58.2	78.4	79.5	69.8
H_2O_2	10.0	12.8	22.8	33.0
	Percentage yield based on *t*-butyl hydroperoxide or H_2O_2			
t-BuOOH	10.1	27.3	55.4	97.4
H_2O_2	1.7	4.4	15.8	45.8

[a]In addition to the indicated amount of *t*-butyl hydroperoxide, each reaction contained 100 mg sodium oleate (329 µmol), 32.4 mL 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)/0.1% Tween 20, pH 7.5, and peroxygenase immobilized on a 142-mm, 0.2 µm Fluoropore membrane. The reaction temperature was 25°C.
[b]In addition to the indicated amount of H_2O_2, each reaction contained 100 mg sodium oleate (329 µmol), 32.4 mL 50 mM acetate/50 mM 2-(*N*-morpholino)ethanesulfonic acid/0.1% Tween 20, pH 5.5, and peroxygenase immobilized on a 142-mm, 0.2 µm Fluoropore membrane. The reaction temperature was 25°C.
[c]Results are the average of two repetitions.

increased 20-fold. Readily available commercial filter holders could accommodate 142-mm (diameter) membranes. These are approximately ninefold higher in area than the 42-mm membranes used for the smaller-scale reactions. It was found that membrane clogging was a problem, and accordingly, ways to reduce the particulate matter in the preparations were sought. The centrifugation force was gradually increased. After each increase, a portion was passed through a Fluoropore membrane, and this was tested for epoxidation activity. It was found that the centrifugation force could be increased from $9000 \times g$ to $16{,}000 \times g$ ($r_{average}$) without large losses in epoxidation activity. In addition, a prefiltration step with a 0.65 μm hydrophilic Durapore membrane was added, as our prior work had shown only minimal binding of peroxygenase to this membrane (9). As noted before, the addition of large amounts of oxidizing agent tended to diminish the activity of peroxygenase, and therefore during the preparation of epoxide, the oxidizing agent was added in steps. The results of selected experiments chosen to illustrate observed trends with 100 mg sodium oleate and *t*-butyl hydroperoxide are shown in Table 1. At the lowest level of added oxidant, Reaction D, the utilization of hydroperoxide was nearly quantitative, giving a 97.4% yield based upon hydroperoxide. However, when the amount of added *t*-butyl hydroperoxide was elevated (Reactions B and C) to achieve better conversion of sodium oleate, the yield of epoxide based upon sodium oleate could be increased only to about 80%, a yield similar to that achieved with 1 mg sodium oleate in our prior research with peroxygenase (9). As illustrated with Reaction A, further increases in *t*-butyl hydroperoxide diminished the yield of epoxide. When hydrogen peroxide was used as the oxidant, the highest yield of epoxide, 33% based on sodium oleate, was obtained at the lowest level of added hydrogen peroxide (Reaction D). As the level of added hydrogen peroxide was increased, the yield of epoxide gradually decreased. Thus, hydrogen peroxide is highly toxic to peroxygenase.

Presumably, further increases in scale are possible with an appropriately engineered bioreactor. Our experiments using hydrogen peroxide were less successful than with *t*-butyl hydroperoxide probably owing to enzymatic deactivation. In earlier work with lipase-generated peracids for epoxide formation, it was also observed that hydrogen peroxide was detrimental to enzymatic activity (14). Through the judicious choice of enzyme support, lipase half-life was increased to an acceptable level. Our selection of the Fluoropore membrane was made by monitoring the conversion of fatty ester hydroperoxide to alcohol epoxide (9). Further investigations of immobilization media using hydrogen peroxide as the oxidant will be necessary to determine if peroxygenase activity can be stabilized to provide better yields of epoxide.

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