

**LIPOXYGENASE CATALYZED HYDROPEROXIDE FORMATION IN
MICROEMULSIONS CONTAINING NONIONIC SURFACTANT**

SUMMARY

Nearly quantitative conversion of linoleic acid to its hydroperoxide was achieved at room temperature ($\sim 24^{\circ}\text{C}$) in microemulsions containing surfactant, water, and organic solvent, using air as the oxygen source, and lipoxygenase from soybeans as the catalyst, providing an excellent route to this strategic intermediate in the synthesis of important industrial products.

INTRODUCTION

Reduction of fatty acid hydroperoxides (FAHP) yields hydroxylated fatty acids. After appropriate modification, such fatty acids could serve as substitutes for ricinoleic acid, the major component of castor oil, which is imported into the USA in large amounts (Vignolo and Naughton, 1991). FAHP may also be subjected to further enzymatic rearrangement. Some of these products are potentially important to the flavor industry (Cardillo *et al.*, 1991). Cleavage routes of FAHP also yield shorter chain fatty materials. These are currently synthesized by industry using expensive ethylene or obtained from imported palm oil (Schirber, 1991). The enzyme lipoxygenase (LOX, EC 1.13.11.12) catalyzes the formation of FAHP from fat and oxygen, and this enzyme is available from both plant and animal sources. However, when formation of FAHP is conducted in aqueous media, in which air is the sole oxygen source, anaerobic conditions are rapidly reached, upon which pentadienyl radicals leak from the active site, leaving LOX in a reduced, inactive state. This form of the enzyme reacts with FAHP to generate alkoxy radicals. The net effect of radical formation is a reduced yield of FAHP due to the formation of "more highly oxidized" byproducts. To overcome this problem, enzymatic transformations have been

conducted with pure oxygen at four atmospheres pressure at 0°C to give an 80% yield of 13-hydroperoxy-9(Z),11(E)-octadecadienoic acid (HPOD) from linoleic acid (LA) (Iacazio *et al.*, 1990). Also, an aqueous reaction medium sparged with oxygen at ambient pressure can give a high yield of FAHP, but many additions of LOX are necessary, as bubbling through aqueous dispersions of fatty acid leads to foaming, resulting in enzyme denaturation. In a recently published investigation of LOX regioselectivity, Antifoam B was used to suppress foaming, but 88 mg of LOX was required to oxygenate 176 mg of substrate at 0°C (Datcheva *et al.*, 1991). Here the ability of a microemulsion containing organic solvent, water, and nonionic surfactant to support HPOD formation by LOX is examined. This reaction system is a derivative of a formulation that was used in a study of β -hydroxysteroid dehydrogenase (Ayala and Mendoza-Hernández, 1990). Although the activity of LOX in a microemulsion has been studied previously (Luisi *et al.*, 1984), the anionic surfactant that was used would be difficult to separate from FAHP.

MATERIALS AND METHODS

Lipoxygenase. LOX-1 from soybean was a commercial preparation (Lipoxidase, Type 1-B) purchased from Sigma (St. Louis, MO). Initial velocity measurements made with aqueous dispersions of linoleic acid (LA) in 200 mM borate buffer (pH 9.0) showed that the activity of LOX was 63 $\mu\text{mol}/\text{min}\cdot\text{mg}$ protein.

Chemicals and materials. LA, cumene hydroperoxide, and the buffers 2-amino-2-methyl-1-propanol (AMP), 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), 2-[N-morpholino]ethanesulfonic acid (MES), N-tris[hydroxymethyl]-methylglycine (TRICINE) were from Sigma (St. Louis, MO). Cyclohexane, 2,2,4-trimethylpentane, 1,1,2-trichlorotrifluoroethane, isopropyl ether, heptane, octane, ammonium iron (II) sulfate hexahydrate (99.997%), 2,6 di-*tert*-butyl-4-methyl-phenol (99+ %), and xylenol orange (sodium salt) were from Aldrich Chemical (Milwaukee, WI). Triton[®] X-35 was a gift from Union Carbide (Danbury, CT). Nonidet[®] P40 was from Fluka Chemical Corp. (Ronkonkoma, New York). Silica gel G thin-layer chromatography (TLC) plates were from Analtech (Newark, DE). Hexane was from Burdick and Jackson (Muskegon, MI). Toluene was from J.T. Baker (Phillipsburg, NJ). Ethyl ether was from Mallinckrodt (Paris, KY).

Hydroperoxide assay. Ultraviolet absorption by the surfactants precluded the use of an assay that followed the development of conjugated diene. HPOD formation was instead measured in the following ways. Initial studies used TLC. Unreacted LA and HPOD were separated on silica gel G TLC plates using a system based upon that developed by Graveland *et al.*, 1968, except that the plates were dipped in boric acid in methanol (5% w/v) and allowed to air dry before two-stage development: diethylether, benzene, ethanol, acetic acid (200:250:10:1 v/v/v/v), development to the top of the TLC plate; air drying; 2,2,4-trimethylpentane, diethylether, acetic acid;

(25:25:1 v/v/v), development to the top of the TLC plate. The TLC plate was sprayed with 60% aqueous sulfuric acid and charred to visualize HPOD.

HPOD formation was also measured using the xylenol orange method (Jiang *et al.*, 1991). The dye reagent consisted of 100 μ M xylenol orange, 250 μ M ammonium iron (II) sulfate hexahydrate, 25 mM H₂SO₄, and 4 mM 2,6-*tert*-butyl-4-methylphenol in methanol-water (90:10 v/v). The sensitivity of the method was increased by adding the H₂SO₄ immediately after the addition of the methanol-water, allowing the color to develop for 45 min, and measuring the absorbance at 590 nm, rather than at 560 nm. Absorbance obeyed Beers law to at least 40 nmol hydroperoxide per ml of dye reagent. Since the sensitivity of the dye reagent was found to decrease with age, it was always prepared on the day of use. Commercial preparations of cumene hydroperoxide and *t*-butyl hydroperoxide (Sigma) were used for calibration.

Microemulsion formation and LOX oxidation. To a 125 ml glass stoppered flask were added surfactant, water containing buffer, and organic solvent. The mixture (approximately 15 ml) was shaken at 250 rpm at room temperature (RT) for 30 minutes. Substrate LA dissolved in ethanol was added, and the mixture was shaken for an additional 15 min. Oxidation was begun by the addition of LOX. To quench the reaction 0.75 ml of 1 N acetic acid was added, followed by the addition of 10 ml isopropyl alcohol. To quantify the level of HPOD the following procedure was used. A 100 μ l sample was withdrawn and diluted with 1.9 ml ethyl alcohol. The xylenol orange reagent (2 ml) was added to three separate 100 μ l aliquots of this alcoholic solution. The absorbance readings of the three solutions were measured, corrected with readings from blanks, and then the mean was calculated. This mean is defined as one datum point. Each datum point was derived from a separately prepared reaction mixture.

RESULTS

Effect of pH. LOX action on LA was examined in a microemulsion containing buffered water adjusted to different pH values. Mixtures of the buffers MES, HEPES, and TRICINE (420 μ mol each) were used, and pH values from 5.0 to 9.0 were examined. In addition to 4.4 ml (244 mmol) buffered water, each microemulsion contained 10 ml (925 mmol) cyclohexane, 100 μ l (1.7 mmol) ethanol, 0.35 g (1.04 mmol) Triton X-35, 0.8 g (1.33 mmol) Nonidet P40, 40 mg (143 μ mol) LA, and 5 mg LOX. Maximal activity was observed with a pH 9.0 buffer, and the activity decreased as the pH was lowered. At pH 7.0 the activity of LOX was only five percent of that observed at pH 9.0. Changing the buffers to a mixture of CAPS and AMPSO allowed the examination of activity at buffer pH values from 8.5 to 11.0. HPOD formed at approximately the same rate from pH 9.0 to pH 10.5, and the rate diminished below pH 9.0 and above pH 10.5. However, even in the pH range of 9.0 to 10.5 the total amount of HPOD synthesized was lower than expected due to the formation of more highly oxidized byproducts as evinced by the appearance of materials that had lower R_f values than HPOD on TLC plates. The microemulsions containing CAPS and AMPSO were viscous, and it is likely that byproduct formation resulted from

poor oxygen uptake due to insufficient mixing. Experiments in which CAPS and AMPSO were examined separately showed that CAPS was responsible for the thickening effect. As will be discussed below, it was found that high LOX activity could be obtained in the presence of only 0.1 g Nonidet P40. The influence of pH upon LOX activity was reexamined in this low surfactant milieu using a mixture of the buffers HEPES, TRICINE, and AMP (420 μmol of each buffer). Buffer pH values from 7.0 to 10.5 were examined, and as was observed before, the optimal pH value was 9.0.

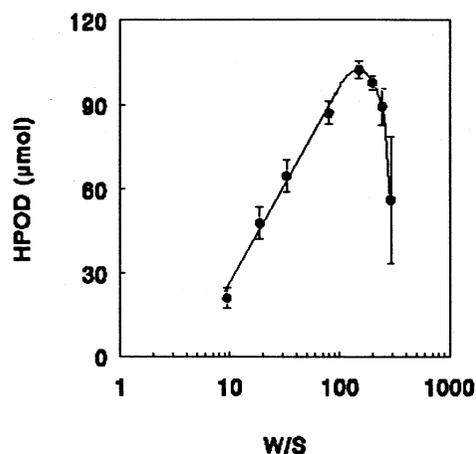


Figure 1. The effect of changes in the ratio of water to surfactant upon the amount of LA oxidized in one hour at RT. The data are the mean \pm SEM of five repetitions.

Effect of Water. Figure 1 shows a semilog plot of HPOD formation at pH 9.0 versus the water-surfactant mol ratio (W/S). In these experiments the amounts of Triton X-35, Nonidet P40, buffer, LA, and LOX were kept constant and at the levels used in the pH study discussed first. Water and cyclohexane were varied to keep the volume fixed. The amount of HPOD formed in one hour was a logarithmic function between W/S values of 4.7 and 145.6. At larger W/S values, the yield of HPOD diminished, and increasing amounts of highly oxidized material were formed. As before, diminished HPOD formation corresponded to increases in the viscosity of the reaction medium. The optimal W/S value for LOX is approximately 10-fold higher than it is for β -hydroxysteroid dehydrogenase (Ayala and Mendoza-Hernández, 1990). Thus, LOX was most active in a relatively polar environment.

Effect of Organic Solvent Composition. Table I shows the influence of various solvents on the amount of HPOD formed in one hour using a relatively high water level (W/S \approx 103). The solvents were saturated with water before their addition to ensure that the level of free water was the same in all experiments. In addition to 10 ml solvent, the reaction media contained 75.3 mg TRICINE buffer (pH 9.0) dissolved in 4.4 ml water, 0.35 g Triton X-35, 0.8 g Nonidet P40, 100 μl ethanol, 40 mg LA and 5 mg LOX. In contrast to the more than 40-fold change in lipase activity observed using similar solvents in a reverse micelle system (Han and Rhee, 1986), LOX activity varied only about 2-fold as the solvent was changed. Thus this observation supports the notion that LOX resides in the aqueous portion of the microemulsion and is

Table I. The influence of solvent composition on the amount of HPOD formed in one hour at RT by LOX in a microemulsion. The data are the mean value \pm SEM for four determinations.

Organic Solvent	HPOD μmol
toluene	128 \pm 18
hexane	66 \pm 14
2,2,4-trimethylpentane	128 \pm 7
1,1,2-trichloro-trifluoroethane	128 \pm 6
cyclohexane	96 \pm 4
isopropylether	120 \pm 12
heptane	87 \pm 9
octane	96 \pm 4

trimethylpentane (TMP), 100 μl (1.7 mmol) ethanol, 40 mg (0.14 mmol) linoleic acid, and 5 mg LOX. As the mol fraction of Nonidet P40 was decreased and that of Triton X-35 increased, the activity of LOX was moderately stimulated. Further increases in the mol fraction of Triton X-35 caused enzyme deactivation. With the optimal mix of Nonidet P40 and Triton X-35 nearly complete conversion to HPOD was achieved in one hour (theoretical maximum: 143 μmol).

However for the purpose of devising a system for the production of HPOD, use of Nonidet P40 alone supports a satisfactory rate of oxidation.

Figure 3 shows a semilog plot of HPOD formation versus the weight of Nonidet P40. The other constituents of the reaction mixture were the same as those described for Figure 2, except that no Triton X-35 was added. The fastest HPOD formation took place at the highest weight of Nonidet P40 used, 1.425 g (2.36 mmol, mol fraction 7.6×10^{-3}). However the amount of Nonidet P40 could be reduced approximately 30-fold to 45 mg (75 μmol), and a satisfactory rate of HPOD formation was still obtained.

relatively sheltered from the organic solvent.

Surfactant Composition. Figure 2 shows a plot of HPOD formation versus the mol fractions of Nonidet P40 and Triton X-35. In addition to the surfactants, the reaction medium contained 4.4 ml (244 mmol) water containing 75.3 mg (0.42 mmol) TRICINE (pH 9.0), 10 ml (60.6 mmol) 2,2,4-

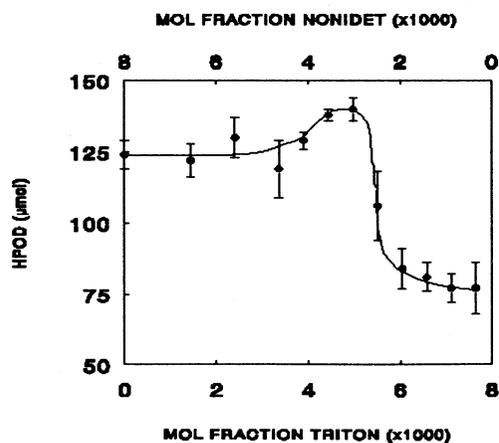


Figure 2. Influence of surfactant composition upon the amount of HPOD formed in one hour at RT. The data are the mean \pm SEM of four repetitions.

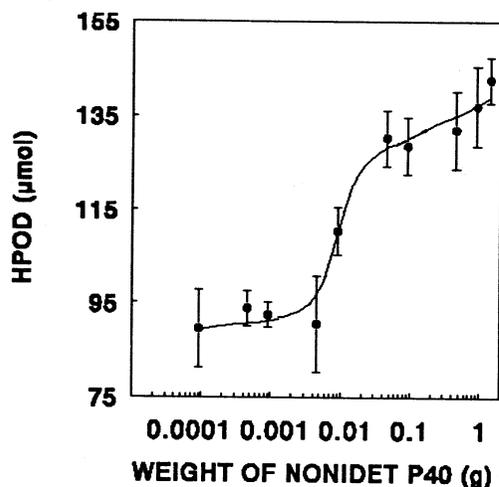


Figure 3. The influence of Nonidet P40 upon the amount of HPOD formed in one hour at RT. The data are the mean \pm SEM of three repetitions.

Further reduction of the level of Nonidet P40 reduced the rate of HPOD formation, and TLC analysis showed that the amount of more highly oxidized byproducts increased.

Final remarks. TLC analysis confirmed that greater HPOD formation took place with less byproduct formation in the microemulsions discussed above than in aqueous media that were not sparged with oxygen, provided that the microemulsions were not too viscous. Studies demonstrated that LOX is not significantly more stable in the microemulsions than in all aqueous media, provided that oxygen is not sparged through the aqueous media. HPOD is also not stabilized in the microemulsions used here, relative to aqueous media. One factor that may contribute toward the enhancement of HPOD formation in microemulsions is greater O_2 solubility in the organic solvent component; in TMP equilibrated with air the O_2 concentration is 3.5 mM, while that in water is only 0.25 mM (Linke, 1965). Also, in earlier studies of nonenzymatic hydrocarbon oxidation it was found that surface-active agents accelerated oxidation, and that at least a portion of this acceleration was due to increased oxygen solubility in the presence of an emulsifier (Elworthy *et al.*, 1968).

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