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Soybean Lipoxygenase-Promoted Oxidation of Free and Esterified Linoleic Acid in the Presence of Deoxycholate

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ABSTRACT: Lipoxygenase (EC 1.13.11.12) catalyzes the reaction between oxygen and polyunsaturated fatty acids to give fatty acid hydroperoxides. Recent work showed that soybean lipoxygenase 1 can oxidize diacylglycerols when deoxycholate is present in the reaction medium. Conditions were sought to maximize 1,3-dilinolein oxidation with a commercial soybean lipoxygenase preparation. It was found that dilinolein was oxidized most rapidly in a multicomponent buffer medium that contained 10 mM deoxycholate between pH 8 and 9. When dilinolein oxidation was conducted in the individual components of the multicomponent buffer, the oxidation rate decreased two- to threefold. Addition of 0.2 M NaCl to one of the components, Tricine buffer, caused a twofold increase in the oxidation rate, demonstrating that high ionic strength is a major factor promoting rapid oxidation in the multicomponent buffer. In the deoxycholate multicomponent buffer, the order of reactivity toward oxidation was monolinolein > methyl linoleate ≈ linoleic acid > dilinolein. Competition experiments in which mixtures of the substrates were presented simultaneously to lipoxygenase in the presence of deoxycholate showed that linoleic acid was the most reactive substrate. When no surfactant was present or when the surfactant was Tween 20, linoleic acid was the most rapidly oxidized substrate. Overall, the results demonstrate that monolinolein and methyl linoleate are just as reactive, or more so, as linoleic acid to oxidation by lipoxygenase under specified reaction conditions. In competition experiments, linoleic acid oxidation predominates, probably because its free carboxyl functionality allows it to be preferentially bound to the active site of lipoxygenase.
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KEY WORDS: Deoxycholate, diacylglycerol, *Glycine max,* hydroperoxide, linoleate, lipoxygenase, monoacylglycerol.

Soybean lipoxygenase (LOX; linoleate, oxygen oxidoreductase, EC 1.13.11.12) generates fatty acid hydroperoxides from polyunsaturated fatty acids (PUFA) and oxygen. In early work, the high-pH form of soybean LOX (LOX 1) was shown to preferentially oxidize PUFA (1). More recently, it was shown that LOX also acted upon esterified fatty acids in phosphoglycerides when a bile salt surfactant was present (2–4). Arachidonylethanolamide, a brain receptor ligand, was reported to be a substrate for soybean LOX (5). A link between fungal elicitor response in plants and the oxidation of phosphatidylcholine by LOX has been demonstrated (6). There are reports concerning the LOX-catalyzed oxidation of phospholipids in liposomes and lipoprotein fractions (7,8).

In contrast to phospholipids, there have been few studies of the action of soybean LOX on neutral lipid esters. Previous studies reported that trilinolein and methyl linoleate are poor substrates for LOX (1,9), but recently it was found that 1,3-dilinolein and 1-stearoyl-2-linoleoyl-*sn*-glycerol were oxidized in the presence of deoxycholate and a commercial preparation of soybean LOX (9). This study was undertaken to optimize the reaction conditions for the oxidation of 1,3-dilinolein and to determine the ability of LOX to oxidize monolinolein and methyl linoleate under these conditions. These studies are important prerequisites for developing industrial-scale processes in which LOX and other related enzymes are used to introduce readily derivatizable oxygen functionality into fats and oils. Such oxygenated derivatives of fats and oils will probably have increased utility as lubricants, polymers, and other value-added products.

MATERIALS AND METHODS

Materials. Soybean (*Glycine max* L. Merr.) LOX (Lipoxidase, Type 1-B), 1-monolinoleoyl-*rac*-glycerol (monolinolein), linoleic acid methyl ester (methyl linoleate), sodium deoxycholate monohydrate, and cumene hydroperoxide were purchased from Sigma (St. Louis, MO). The 1,3 isomer of dilinolein was obtained from Nu-Chek-Prep (Elysian, MN). The sodium salt of xylenol orange was supplied by Aldrich (Milwaukee, WI). Water was purified to a resistance of 18 mΩ · cm in a Barnstead (Dubuque, IA) NANOpure system. All other reagents were of the highest purity available.

Hydroperoxide formation. The substrate (6 μmol linoleoyl residue), dissolved in methylene chloride, was placed in a 10-mL Erlenmeyer flask, and the methylene chloride was evaporated under a stream of nitrogen. To the flask were added 0.2 mL 100 mM deoxycholate and 1.8 mL aqueous buffer (pH

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8.0), consisting of a mixture of four buffers, each at 0.1 M: 2-amino-2-methyl-1-propanol hydrochloride (AMP), *N*-tris(hydroxymethyl)-methylglycine (TRICINE), *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), and 2-(*N*-morpholino)-ethanesulfonic acid (MES). After allowing the reaction medium to incubate at 15°C with agitation at 250 rpm for 30 min, the indicated amount of LOX was added to initiate oxidation. Enzymatic oxidation was quenched with 400 μL 1 M citric acid, and fatty hydroperoxide was extracted with two 1.5-mL aliquots of chloroform/methanol (2:1, vol/vol). After removing the solvent under a stream of nitrogen, the hydroperoxide was redissolved in 3.0 mL ethanol.

Spectrophotometric hydroperoxide assay. The level of hydroperoxide was determined spectrophotometrically by the xylenol orange method as described previously (10,11). In this method, iron (II) is oxidized by hydroperoxide, and the resulting iron (III) forms a colored complex with xylenol orange. Freshly diluted commercial cumene hydroperoxide was used to prepare a calibration curve each day. All results were corrected for nonenzymatic oxidation by subtracting the values obtained from controls that received no LOX.

LOX assay by high-performance liquid chromatography (HPLC). The rate of oxidation was followed by measuring the decrease in the area of the peak corresponding to the substrate. Reaction products were separated on an Alltech (Deerfield, IL) C_{18} hydroxyethylmethacrylate (HEMA) column (250 × 4.6 mm) installed on a Waters (Milford, MA) LCM1 HPLC instrument. The detector was a Varex evaporative light-scattering detector MK III (Alltech) operated at a temperature of 48°C, with N_2 as the nebulizing gas at a flow rate of 1.3 mL/min. The mobile phase had the following composition and gradient: methanol/10 mM aqueous trifluoroacetic acid (TFA) (86:14), 0–4 min; methanol/10 mM TFA (90:20), 4–8 min; acetonitrile/methanol/10 mM TFA (30:65:5), 8–10 min; acetonitrile/methanol/10 mM TFA (51:48:1), 10–25 min. The flow rate was 1 mL/min. The time course of oxidation was monitored as described previously (12).

RESULTS AND DISCUSSION

Optimizing 1,3-dilinolein oxidation. Short assay times (15 min) and suboptimal concentrations of LOX were used so that the assay results would approximate initial oxidation rates. Figure 1 shows the influence of pH upon the amount of 1,3-dilinolein oxidized within 15 min in the deoxycholate-containing buffered system. The activity profile was the typical bell-shaped curve that is seen with many enzymes. The activity of LOX on dilinolein was optimal between pH values of 8 and 9 and decreased as the pH value of the medium was either reduced or raised from this range. Also plotted in Figure 1 is the pH profile of trilinolein oxidation in deoxycholate containing buffer (9). The trilinolein pH profile is similar to the profile of dilinolein, except that the trilinolein profile is slightly broader at pH < 7.

Figure 2 shows the effect of deoxycholate concentration on the amount of dilinolein oxidized in 15 min. The amount of hydroperoxide produced increased almost linearly as the deoxycholate concentration increased to 10 mM. At 10 mM deoxycholate, the oxidation rate had been increased sixfold over that obtained in a medium containing no deoxycholate. Further increases in the amount of deoxycholate caused a small decrease in the amount of oxidation. The optimal deoxycholate concentration for trilinolein oxidation was reported to be 5 mM (9), while that for phosphoglyceride oxidation was 10 mM (2).

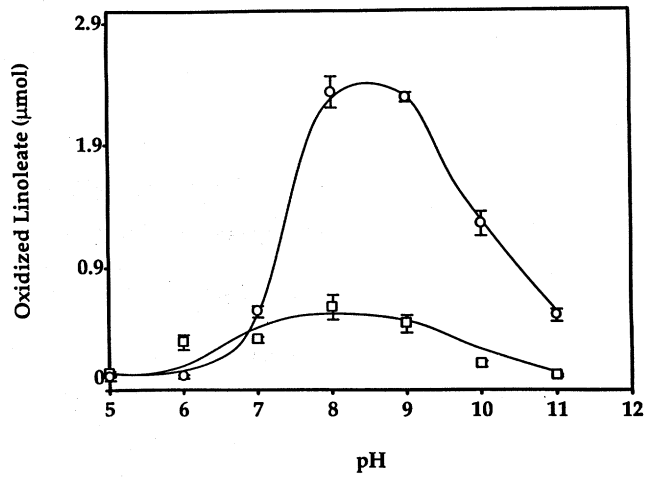

FIG. 1. Influence of pH on 1,3-dilinolein (○) and trilinolein (□) oxidation by lipoxygenase (LOX) in 10 mM deoxycholate. Dilinolein assays were performed as described in the Materials and Methods section with 75 μg LOX, and the hydroperoxide level was determined with the Xylenol Orange method. The data are the means ± SEM for three determinations. The data for trilinolein oxidation are from Piazza and Nuñez (Ref. 9).

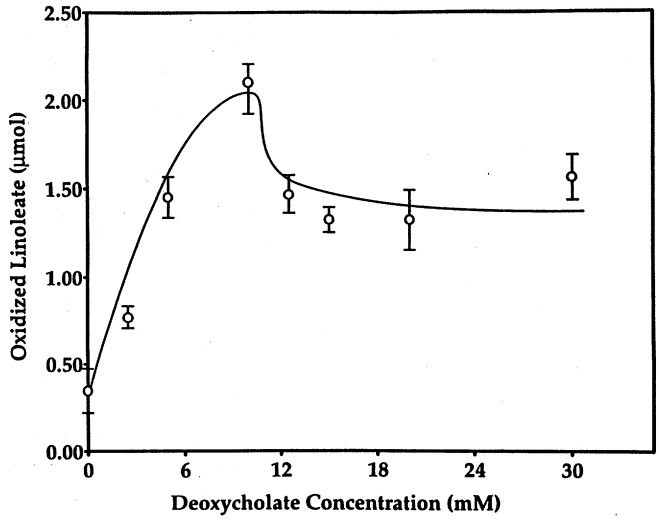

FIG. 2. Influence of deoxycholate concentration on the oxidation of 1,3-dilinolein by LOX. Assays were performed as described in the Materials and Methods section with 50 μg LOX. The hydroperoxide level was determined with the Xylenol Orange method. The data are the means ± SEM for seven determinations. See Figure 1 for abbreviation.

The influence of LOX concentration upon dilinolein oxidation in 15 min was also investigated (Fig. 3). The reaction conditions were identical to those used in Figure 1 at pH 8. The degree of oxidation increased as the level of LOX increased up to 1.25 mg (~0.6 mg protein). Further increases in LOX caused the amount of oxidized linoleate to decrease somewhat. This decrease is probably the result of the anaerobic action of LOX upon the hydroperoxide initially produced (13).

The experiments discussed previously were conducted in the multicomponent buffer described in the Materials and Methods section. When oxidations were conducted in 0.1 M AMP, HEPES, MES, or TRICINE, the extent of dilinolein oxidation in 15 min was reduced approximately two- to threefold. The possibility that this decrease is due in part to diminished ionic strength is shown by the data in Figure 4. Here different concentrations of NaCl were used with a 0.1 M TRICINE buffer. The extent of oxidation remained nearly constant to 10 mM NaCl, then increased about twofold at 200 mM NaCl. Further increases in the NaCl concentration caused the extent of oxidation to diminish. The increased oxidation from lower NaCl concentrations may be due to enhanced complex formation between dilinolein and deoxycholate in a more polar environment. This stimulation is overcome by LOX inhibition at high concentrations of NaCl. In 0.1 M TRICINE containing 200 mM NaCl, the rate of dilinolein oxidation was approximately 90% of that in the multicomponent buffer. Thus, high ionic strength is the primary reason why oxidation occurs more rapidly in the multicomponent buffer.

Oxidation rates of individual substrates. Having established optimal reaction conditions for dilinolein, we tested other linoleate esters and compared their extent of oxidation in 15 min to that of free linoleic acid (Table 1). Monolinolein was oxidized over twice as fast as dilinolein and slightly faster than linoleic acid. Data from Piazza and Nuñez (9) on the oxidation of trilinolein in deoxycholate are included in Table 1 to demonstrate that the addition of a third linoleoyl residue on the glycerol backbone causes a further decrease in reactivity. The methyl ester of linoleic acid was oxidized at approximately the same rate as linoleic acid. In contrast, earlier work that was performed with Tween 20 (polyoxyethylene-sorbitan monlaurate) without deoxycholate showed that the methyl ester of linoleic acid was a poor substrate for LOX (1). Because the results obtained in deoxycholate were at such variance with these earlier results, assays were repeated in the presence of no added surfactant and in Tween 20. Table 1 shows that, without surfactant or in the presence of Tween 20, the oxidation of linoleic acid was accelerated, but monolinolein and methyl linoleate were oxidized at significantly reduced rates compared to those obtained in deoxycholate. No oxidation of dilinolein could be detected in the presence of Tween 20 or without surfactant.

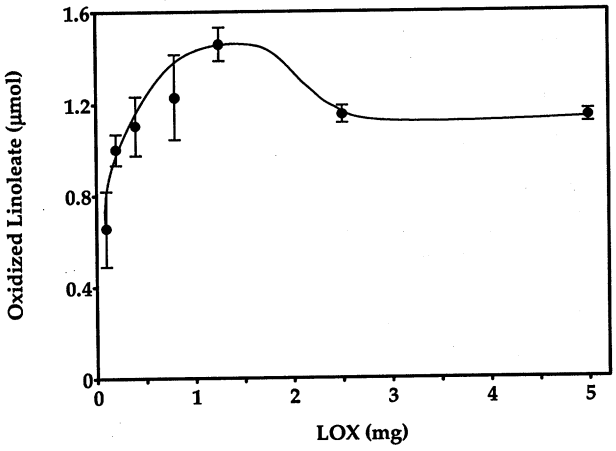

FIG. 3. Dependence of 1,3-dilinolein oxidation upon the level of added LOX. Assays were performed as described in the Materials and Methods section. The hydroperoxide level was determined with the Xylenol Orange method. The data are the means ± SEM for five determinations. See Figure 1 for abbreviation.

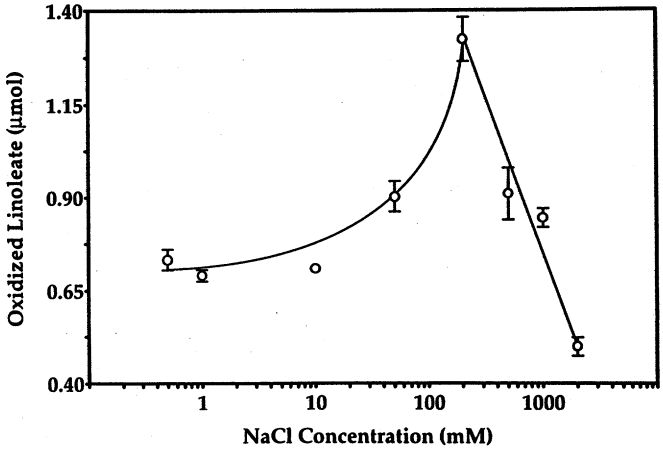

FIG. 4. The effect of NaCl on 1,3-dilinolein oxidation by LOX in 10 mM deoxycholate. Assays were performed as described in the Materials and Methods section with 75 µg LOX, except the buffer consisted of 0.1 M *N*-tris(hydroxymethyl)-methylglycine, pH 8.0. The hydroperoxide level was determined with the Xylenol Orange method. The data are the means ± SEM for four determinations. See Figure 1 for abbreviation.

TABLE 1
Relative Amounts of Oxidized Linoleate Formed in 15 min by Lipoxygenase (LOX)

	Relative amount of oxidation[a]		
Substrate	Deoxycholate[b]	Without surfactant	Tween 20[c]
Linoleic acid	100 ± 6	186 ± 1	195 ± 7
Methyl linoleate	90 ± 8	28 ± 1	12 ± 1
1-Monolinolein	126 ± 6	11 ± 1	72 ± 3
1,3-Dilinolein	55 ± 4	NS[d]	NS
Trilinolein	3 ± 1[e]		

[a]Assays of each substrate were conducted separately as described in the Materials and Methods section with 25 µg lipoxygenase (LOX) per assay. The amount of linoleic acid oxidized in deoxycholate was 0.986 µmol. Data are the means ± SEM for three determinations.
[b]The deoxycholate concentration was 10 mM.
[c]The Tween 20 concentration was 0.25% (vol/vol) (Ref. 18).
[d]Results were not significantly different from zero.
[e]Data from Reference 9. Assay conditions were identical to those used here, except 200 µg LOX was added.

Relative oxidation rates of substrate mixtures. When the oxidation of dilinolein, methyl linoleate, and monolinolein was followed in the presence of linoleic acid, their oxidation rates were reduced relative to that of linoleic acid (compare Table 1 and Table 2, entries 1, 2, and 3). However, even in the presence of linoleic acid, monolinolein was still the most rapidly oxidized ester substrate. When 1,3-dilinolein and methyl linoleate were oxidized together in the presence of linoleic acid, their relative oxidation rates were reduced further, although the amount of LOX was increased proportionately (Table 2, entry 4). The combination of 1,3-dilinolein and monolinolein was also oxidized in the presence of linoleic acid (Table 2, entry 5). Each substrate was oxidized at approximately the same relative rate as when they were separately assayed against linoleic acid.

Work by several research groups has demonstrated the importance of the methyl terminus of the fatty acid chain in directing the site of hydrogen removal by L[+2]-type LOX, such as the high-pH form of soybean LOX (14–16). However, the rate and specificity of hydroperoxide formation are also influenced by the presence of the free carboxylic residue in the substrate. For example, at high pH values (13*S*)-(9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid is the major product of the action of soybean LOX on linoleic acid. As the pH is lowered, and the carboxylic acid moiety becomes increasingly protonated, increasing amounts of the 9-hydroperoxide isomer are formed (17). Additionally, LOX oxidizes the structural analogues of naturally occurring fatty acids more slowly if the carboxylic acid group has been displaced by additional, intervening methylene groups, even if their 1,4-pentadienyl moiety is located in such a way that a hydroperoxide can form at ω6 (14).

The data found in Tables 1 and 2 show that the presence of a free carboxyl group on the substrate influences the magnitude of the interaction between the substrate and LOX. If the order of reactivity of the substrates were determined solely by the ability of deoxycholate to solubilize the substrates, then a change in the order would not be expected, depending on whether the substrates were assayed separately or together. Since linoleic acid is always oxidized more rapidly in the competition experiments as shown in Table 2, linoleic acid must be preferentially bound by LOX.

In conclusion, the results demonstrate that methyl linoleate, as well as mono- and diacylglycerol that contain linoleoyl residues, are rapidly oxidized by LOX in buffers that contain deoxycholate surfactant. The oxidized products are useful chemical intermediates for further transformation to emulsifiers and lubricants with enhanced hydrophilic properties, and the oxygenated methyl ester could also be used directly as an octane enhancer in biodiesel preparations.

TABLE 2
Relative Oxidation Rate of Substrate Mixtures by LOX in Deoxycholate

Substrate mixtures	Relative oxidation rates[a]
1. Linoleic acid	100[b]
1,3-Dilinolein	36 ± 2
2. Linoleic acid	100[b]
Methyl linoleate	39 ± 3
3. Linoleic acid	100[b]
1-Monolinolein	75 ± 2
4. Linoleic acid	100[c]
1,3-Dilinolein	10 ± 2
Methyl linoleate	18 ± 2
5. Linoleic acid	100[c]
1,3-Dilinolein	36 ± 9
1-Monolinolein	65 ± 4

[a]Each determination consisted of nine independent assays that were quenched at 5 min intervals between 0 and 40 min. Assays were prepared as described in the Materials and Methods section, except each assay initially contained 6 μmol linoleoyl residues of each substrate and diolein, as an internal standard. Relative oxidation rates were calculated by the method of Schellenberger *et al.* (Ref. 19). Data are the means ± SEM for three determinations. See Table 1 for abbreviation.
[b]Each time point sample contained 75 μg LOX.
[c]Each time point sample contained 113 μg LOX.

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