# Oxidation of Acylglycerols and Phosphoglycerides by Soybean Lipoxygenase

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**ABSTRACT:** Lipoxygenase (EC 1.13.11.12) catalyzes the incorporation of oxygen into polyunsaturated fatty acids, resulting in the formation of their corresponding hydroperoxides. The ability of a commercial preparation of soybean (*Glycine max* L. Merr.) lipoxygenase to catalyze the oxidation of acylglycerols and phosphoglycerides was investigated. The oxidation rate of trilinolein increased nearly 100% when 5 mM deoxycholate was added to the reaction medium. With further increases in the concentration of deoxycholate, the oxidation rate decreased slightly. The pH profile of trilinolein oxidation was bell-shaped. The rate of oxidation was maximal at pH 8, and it decreased to near zero at pH 5 and pH 11. Even under optimal conditions, the rate of trilinolein oxidation was only 3% of that of linoleic acid, and analysis of time course data showed that, at most, 15% of available linoleate was oxidized. In contrast to the slow rate of trilinolein oxidation, tested phosphoglycerides and diacylglycerols were oxidized at moderate rates. The rate of phosphoglyceride oxidation depended upon the structure of the polar head group and varied between 7–28% of the rate of linoleic acid oxidation. Diacylglycerols reacted at a rate that was 40% of that of linoleic acid. Analysis of the time course of 1,3-dilinolein oxidation showed that as much as 67% of the available linoleate could be converted to the corresponding hydroperoxide. Analyses by high-performance liquid chromatography revealed that more than 20% of the 1,3-dilinolein was converted to unidentified products that are not hydroperoxides. *JAOCS 72,* 463–466 (1995).

**KEY WORDS:** Acylglycerol, deoxycholate, fatty acid, *Glycine max,* hydroperoxide, lipoxygenase, phosphoglyceride.

Soybean lipoxygenase (LOX; linoleate, oxygen oxidoreductase, EC 1.13.11.12) is a commercially available enzyme that catalyzes the reaction of oxygen and fatty acids that contain a 1,4-diene unit to give fatty acid hydroperoxides. Although LOX suffers from a time-dependent inactivation (1), yields of linoleic acid hydroperoxide in excess of 80% can be achieved with a single addition of LOX (2). It has recently been shown that immobilized LOX has enough stability to be utilized as a synthetic reagent for the selective modification of fatty acids (3). The available evidence indicates that 15(*S*)-lipoxygenases, such as soybean LOX, recognize and bind their substrates through the methyl end, and therefore, modification to the carboxylic end of the substrate should have a minimal impact upon substrate reactivity (4). In support of this hypothesis, it was recently demonstrated that LOX acts on polyunsaturated phosphoglycerides, provided that a bile salt surfactant is present (5–8). Phosphoglyceride oxidation was highly positionally and stereochemically specific and therefore was not merely an enzyme-promoted radical-mediated autoxidation (6). Early studies of soybean LOX showed that trilinolein was a poor substrate (9). That phosphoglycerides can be good substrates for LOX in the presence of a bile salt raises the question as to whether acylglycerols can also be acted upon rapidly under these reaction conditions. Therefore, we have reexamined acylglycerol oxidation by LOX in the presence of the bile salt deoxycholate to determine whether LOX oxidizes acylglycerols as well as phosphoglycerides under these conditions. These studies are important prerequisites to possible industrial-scale processes in which lipoxygenase and other enzymes are used to introduce readily derivatizable oxygen functionality into fats and oils to increase their utility in lubricants and other value-added products.

## MATERIALS AND METHODS

*Materials.* Soybean (*Glycine max* L. Merr.) LOX (Lipoxidase, Type 1-B), sodium deoxycholate monohydrate, and cumene hydroperoxide were purchased from Sigma (St. Louis, MO). Except as indicated, acylglycerols and linoleic acid were also purchased from Sigma. Phosphoglycerides were from Avanti Polar-Lipids (Alabaster, AL). Biomol (Plymouth Meeting, PA) supplied 1-stearoyl-2-linoleoyl-*sn*-glycerol. The sodium salt of xylenol orange was purchased from Aldrich (Milwaukee, WI). Thin-layer chromatography (TLC) plates were purchased from Analtech (Newark, DE). 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.* An aliquot of the substrate (6 μmol linoleoyl residues) dissolved in chloroform was placed in a 10-mL Erlenmeyer flask, and the chloroform was evaporated under a stream of dry nitrogen. In addition to substrate, the reaction medium contained 0.2 mg LOX, 0.2 mL 100 mM

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deoxycholate, and 1.8 mL aqueous buffer, consisting of an equal 0.1 M mixture (0.4 M total) of 2-amino-2-methyl-1-propanol hydrochloride, *N*-tris(hydroxymethyl)-methylglycine (Tricine), *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), and 2-(*N*-morpholino)ethanesulfonic acid (MES). Oxidation was conducted at 15°C with agitation at 250 rpm for the indicated time. The reaction was quenched with 400 µL 1 M citric acid, and fatty acid hydroperoxide was extracted within 1 h 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.

*Hydroperoxide assay.* The level of hydroperoxide was determined spectrophotometrically by the xylenol orange method as described before (10). Freshly diluted commercial cumene hydroperoxide was used for preparing a calibration curve each day. All results were corrected by subtracting readings from controls that were quenched with citric acid immediately after the addition of LOX. Confirmation of the readings from the xylenol orange method was accomplished with TLC on pre-adsorbent HPTLC-HL, 10 × 10 cm silica gel plates that had been dipped in 5% boric acid in methanol and allowed to dry before sample application. Development was achieved with a two-stage double development system: toluene/ethyl acetate/diethyl ether/acetic acid (75:13:10:1.2, by vol); hexane/diethyl ether/formic acid (64:16:2, vol/vol/vol). The TLC plate was allowed to air-dry between solvent systems. After development, the TLC plate was sprayed with 60% aqueous sulfuric acid and charred to visualize the hydroperoxide and starting material.

*Assay for 1,3-dilinolein oxidation.* In addition to the xylenol orange method, the rate of oxidation of 1,3-dilinolein was followed by measuring its decrease with time by using high-performance liquid chromatography (HPLC). The reaction mixture was identical to that described above for trilinolein oxidation, except that 4 µmol 1,3-diolein was added as an internal standard. Reactions were quenched and extracted at 5-min time intervals over 0 to 40 min. Reverse-phase HPLC was conducted with an Alltech (Deerfield, IL) C18 hydroxyethylmethacrylate (HEMA) column (250 × 4.6 mm), installed on a Hewlett-Packard Series 1050 instrument equipped with a Varex (ELSD IIA) evaporative light-scattering detector (Palo Alto, CA). The mobile phase had the following composition and gradient: methanol/water (86:14), 0–5 min; (90:10), 5–8 min; methanol/acetonitrile/water (65:30:5), 10–13 min; (48:51:1), 13 min. Under these conditions, the oxidized products did not interfere with the measurement of the remaining substrate. Peak areas were converted to molar values by means of a calibration curve prepared by injection of 1,3-dilinolein solutions of known concentration.

*Time course analysis.* Time courses of acylglycerol oxidation were analyzed by nonlinear regression with the program Abacus, which is based upon the Gauss-Newton iterative method (11). Iterative analysis was carried out until the minimum in the root mean square error was found. The mathematic expression that describes the time course of product formation is as follows (Eq. 1):

$$P_{obs} = \frac{P_{max} k_1^n t^n}{1 + k_1^n t^n} \quad [1]$$

where $P_{obs}$ is the amount of product observed at time $t$, $P_{max}$ is the maximum amount of product formed, $k_1$ is the rate of reaction at one-half $P_{max}$, and $n$ is the cooperativity parameter that is constrained to integer values. The reciprocal of $k_1$ yields the time needed to generate one-half $P_{max}$ and is therefore the reaction half time.

## RESULTS AND DISCUSSION

Trilinolein oxidation products were analyzed by TLC. The primary oxidation product ($R_f = 0.6$) ran immediately behind trilinolein ($R_f = 0.74$) and was well separated from linoleic acid ($R_f = 0.52$) and its oxidation product. That oxidized linoleic acid was not detected demonstrates that the oxidation of esterified fatty acid is the primary route to hydroperoxide.

The pH rate profile of LOX-catalyzed oxidation of trilinolein was determined in the presence of 10 mM deoxycholate and is shown in Figure 1. The profile was bell-shaped, with the highest rate of oxidation observed at approximately pH 8. At pH 5 and pH 11, almost no oxidation was observed.

The influence of the bile salt surfactant deoxycholate upon trilinolein oxidation was examined at pH 8, and the results are shown in Figure 2. Trilinolein oxidation was observed even when no deoxycholate was present, although when 5 mM deoxycholate was added, the rate of oxidation nearly doubled. With further increases in deoxycholate, the oxidation rate decreased slightly. The rate of soybean LOX-catalyzed oxidation of phosphatidylcholine was reported to be maximal in the presence of 10 mM deoxycholate (5).

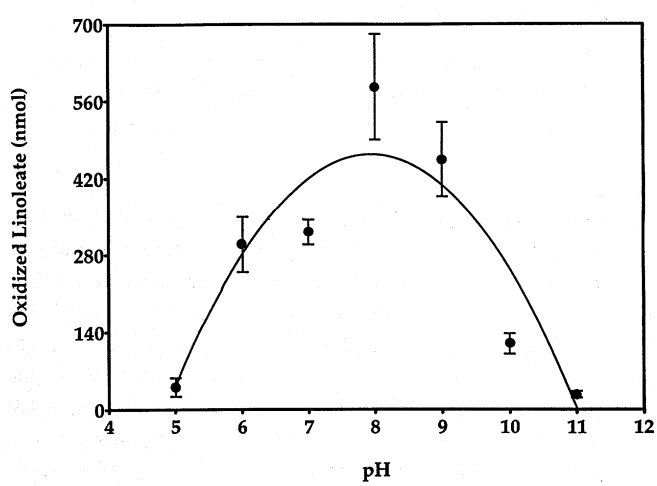


**FIG. 1.** Influence of pH on trilinolein oxidation by lipoxygenase (LOX). Each assay contained 2 µmol trilinolein, 10 mM deoxycholate, and 0.1 mg LOX. Assays were conducted for 2 h at 15°C. The data are the mean ± SEM for five to twelve determinations.

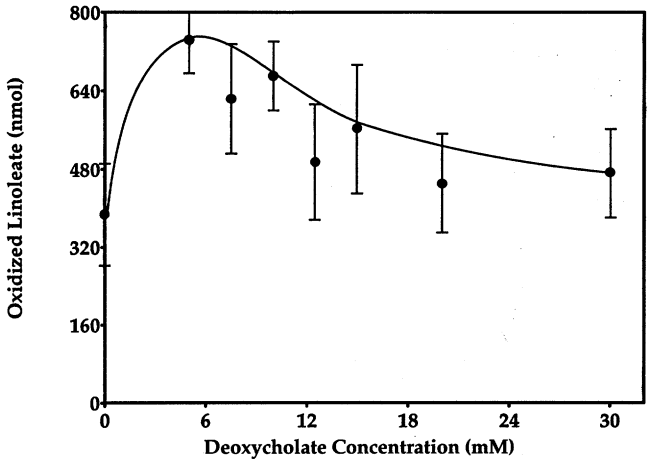


**FIG. 2.** Effect of deoxycholate concentration upon trilinolein oxidation by lipoxygenase (LOX). Each assay contained 2 μmol trilinolein and 0.1 mg LOX. Assays were conducted at pH 8.0 for 2 h at 15°C. The data are the mean ± SEM for six determinations.

The time course of trilinolein oxidation was determined and is shown in Figure 3. The oxidation rate was rapid during the first 20 min but then decreased rapidly. The time course of trilinolein oxidation was examined with nonlinear regression analysis. The best curve fit to Equation 1 was obtained with the cooperativity parameter, *n*, equal to one. Potentially, 0.92 μmol of available linoleate (6 μmol) could be converted to hydroperoxide, which is equivalent to a 15% conversion.

One explanation for the limited production of hydroperoxide was that LOX became inactivated during the oxidation process through a mechanism that is observed even when linoleic acid is the substrate (1). To test this possibility, a second addition of freshly prepared LOX was made one hour after the first addition. Samples were withdrawn after an additional 30 min and one hour, analyzed for hydroperoxide content, and compared to controls that received only one addition of LOX. Analyses conducted 30 min after the second addition of LOX revealed a slightly elevated level of hydroperoxide compared to the control level. After one hour, regardless of whether one or two additions of LOX were made, the levels of hydroperoxide in both treatments were the same within experimental error (n = 6) . Thus, low conversions to oxidized product are not merely the result of substrate-independent LOX inactivation.

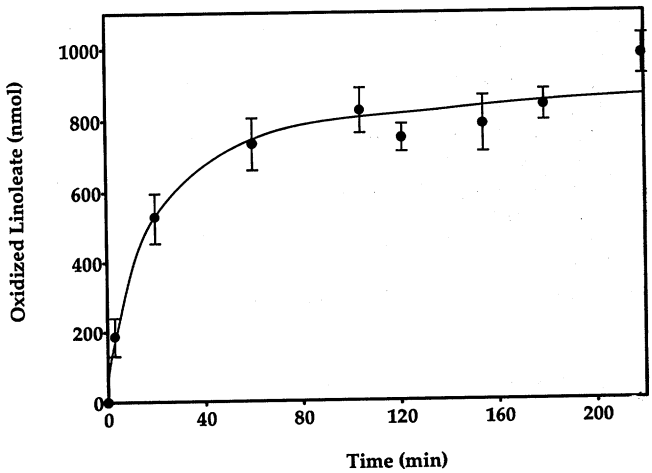


**FIG. 3.** Time course of trilinolein oxidation by lipoxygenase (LOX) as determined by the xylenol orange method. Each assay contained 2 μmol trilinolein, 10 mM deoxycholate, and 0.2 mg LOX. Assays were conducted at pH 8.0 for the indicated time at 15°C. The data are the mean ± SEM for seven determinations.

Table 1 shows the relative rate of LOX-catalyzed oxidation of linoleic acid and several phosphoglycerides and acylglycerols. All of the tested phosphoglycerides were substrates of LOX, although their rates of oxidation were considerably reduced compared to that of linoleic acid. The phosphoglyceride substrate that was hydrolyzed the fastest, phosphatidylserine (1,2-diL, 3-*P*-serine), reacted at 28% of the rate of linoleic acid. A comparison of the two tested phosphatidylcholine species (1-L, 2-S, 3-*P*-choline and 1-S, 2-L, 3-*P*-choline) suggested that a slight preference exists for the oxidation of a primary linoleate residue. However, *t*-test analysis of the data demonstrated that the difference in the observed rates was not statistically significant. Trilinolein was oxidized at a low rate compared to linoleic acid. Substitution of oleate and stearate for linoleate at positions 2 and 3 (1-L, 2-O, 3-S) resulted in a slightly better triacylglycerol substrate. However, the removal of one fatty acid to form the diacylglycerol resulted in substrates (1-L, 3-L and 1-S, 2-L) that reacted more rapidly, approximately 40% as fast as linoleic acid.

Figure 4 shows the time course of 1,3-dilinolein oxidation by LOX. The extent of oxidation was determined by the

**TABLE 1**
**Relative Rate of Oxidation of Linoleic Acid and Several Phosphoglycerides and Acylglycerols Catalyzed by LOX**

| Substrate | Oxidation rate (%)[a] |
|---|---|
| Linoleic acid | 100 |
| 1,2-diL, 3-*P*-serine[b] | 28 ± 8 |
| 1-L, 2-S, 3-*P*-choline | 12 ± 4 |
| 1-S, 2-L, 3-*P*-choline | 7 ± 3 |
| 1-P-, 2-L, 3-*P*-ethanolamine | 13 ± 2 |
| 1-P, 2-L, 3-*P* | 15 ± 5 |
| Trilinolein | 3 ± 1 |
| 1-L, 2-O, 3-S | 11 ± 4 |
| 1-L, 3-L | 40 ± 5 |
| 1-S, 2-L | 43 ± 4 |

[a]Each assay contained 6 μmol linoleate, 10 mM deoxycholate, and 0.2 mg lipoxygenase (LOX). Assays were conducted at pH 8.0 for 15 min at 15°C. The data are mean ± SEM for five to ten determinations.
[b]Substrate abbreviations: 1,2-diL, 3-*P*-serine = 1,2-dilinoleoyl-*sn*-glycero-3-phospho-L-serine; 1-L, 2-S, 3-*P*-choline = 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; 1-S, 2-L, 3-*P*-choline = 1-stearoyl, 2-linoleoyl-*sn*-glycero-3-phosphocholine; 1-P, 2-L, 3-*P*-ethanolamine = 1-palmitoyl-2-linoleoyl-*sn*-glycerol-3-phosphoethanolamine; 1-P, 2-L, 3-*P* = 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphate; 1-L, 2-O, 3-S = 1-linoleoyl-2-oleoyl-3-stearoyl-*rac*-glycerol; 1-L, 3-L = 1,3-dilinolein; and 1-S, 2-L = 1-stearoyl-2-linoleoyl-sn-glycerol.

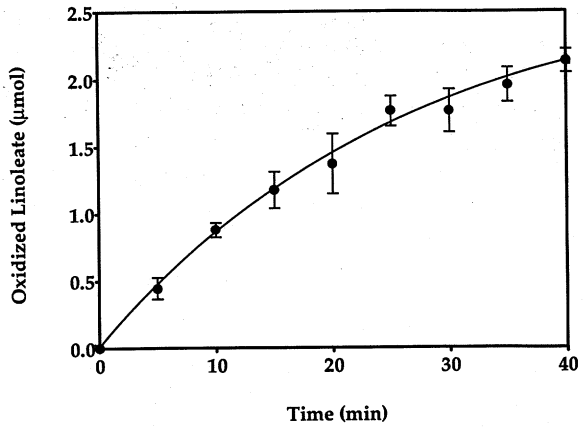


**FIG. 4.** Time course of 1,3-dilinolein oxidation by lipoxygenase (LOX) as determined by the xylenol orange method. Each assay contained 3 µmol 1,3-dilinolein, 10 mM deoxycholate, and 0.2 mg LOX. Assays were conducted at pH 8.0 for the indicated time at 15°C. The data are the mean ± SEM for four determinations.

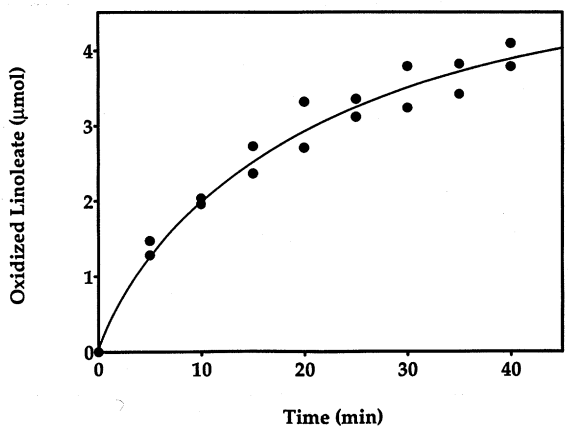


**FIG. 5.** Time course of 1,3-dilinolein oxidation by lipoxygenase as determined by high-performance liquid chromatography to follow the rate of disappearance of the substrate. Reaction conditions were as described in Figure 4.

xylenol orange method, which measures hydroperoxide formation. The curve in Figure 4 shows the results of nonlinear regression analysis by using Equation 1 with $n$ equal to one. The maximum amount of product that can be formed was 4.02 µmol or 67% conversion, a value considerably higher than that obtained with trilinolein.

Figure 5 also shows the time course of 1,3-dilinolein oxidation by LOX, with the extent of oxidation determined by the peak area corresponding to 1,3-dilinolein after HPLC analysis. The maximum degree of oxidation was 5.39 µmol, equivalent to about 90% conversion to product. The reason for the discrepancy between the results obtained with the xylenol orange method and those obtained with HPLC is that slightly more than 20% of the 1,3-dilinolein was converted to products that are not hydroperoxide(s). Both HPLC and TLC revealed the formation of products that did not correspond to the mono- and dihydroperoxides of 1,3-dilinolein. The structural nature of these other products is not known, but their formation cannot simply be the consequence of the anaerobic LOX cycle, because under the reaction conditions used, oxygen was not depleted. However, steric constraints may be causing 1,3-dilinolein to bind to LOX in a nonproductive or a less productive mode, allowing an increase in the rate of nonspecific radical reactions, even when oxygen is available.

In conclusion, the results demonstrate that, although trilinolein is a poor substrate, diacylglycerols are oxidized relatively rapidly by LOX when deoxycholate is present. These oxidized diacylglycerols might find utility as intermediates in the synthesis of triacylglycerols that contain specifically oxidized fatty esters or, after appropriate chemical modification, as emulsifiers with enhanced hydrophilic properties.

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