

Analysis of Lipoxygenase Kinetics by High-Performance Liquid Chromatography with a Polymer Column

ABSTRACT: Soybean lipoxygenase (LOX; EC 1.12.11.12) catalyzes the oxygenation of polyunsaturated fatty acids, acylglycerols and phosphoglycerols, producing a regio- and enantiospecific hydroperoxide product. The goal of this work was to measure the relative rate of LOX-catalyzed oxidation of mixtures of lipids containing linoleate, using high-performance liquid chromatography (HPLC) and a light-scattering detector (LSD). Previous literature suggested that reversed-phase HPLC with silica-based columns could be used for the separation of individual fatty acids, acylglycerols, phosphoglycerides and their oxidation products. However, these columns produced ineffective separations of phosphoglycerides unless choline chloride and a strong base, such as KOH, are present in the mobile phase. Such modifiers precluded the use of the LSD. It was found that a reversed-phase column based upon an organic polymer support, rather than on silica, was able to separate these mixtures with a ternary solvent gradient of methanol/water/acetonitrile without the need for the addition of modifiers. The oxidation time course of a mixture of linoleic acid, trilinolein and 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine was followed using the developed HPLC method. The results showed that trilinolein and phosphatidylcholine reacted at one-tenth the rate of linoleic acid. The diacylglycerol, 1,3-dilinolein, was oxidized at a rate that was approximately 40% that of linoleic acid, with the formation of mono- and dihydroperoxides as well as other unidentified products.

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The enzyme, lipoxygenase (LOX; EC 1.13.11.12), catalyzes the oxidation of polyunsaturated fatty acid containing a *cis*, *cis*-1-4-pentadiene moiety, producing fatty acid hydroperoxides. It is known that LOX from a variety of natural sources produces a single major product (1,2). For example 13(*S*)-hydroperoxy-9,11-octadecadienoic acid results from the action of soybean LOX upon linoleic acid (LA) (3). Recently it was shown that immobilization of LOX enhances its stability, thereby increasing its potential as a synthetic reagent for the

selective oxidation (4,5). In addition to free fatty acid substrates, early studies showed that polyunsaturated acylglycerols undergo oxidation with soybean LOX (6-8). More recently, it was shown that this enzyme catalyzes the oxidation of phosphatidylcholine, provided that a bile salt surfactant is present (9). Structural analysis of the products illustrated the high positional and stereochemical specificity of this reaction (10). Although prior work has shown that LOX acts upon fatty acids, acylglycerols and phosphoglycerides, the substrate specificity of LOX has never been determined with a bile salt present in the reaction medium.

To measure enzyme specificity, Schellenberger *et al.* (11) have developed a statistical method for the analysis of reactions of competitive substrates. To use this method, the relative concentrations of all substrates or products must be followed over time by an analytical technique. For the analysis of LOX substrates and products, high-performance liquid chromatography (HPLC) is an excellent option. There is a vast volume of HPLC methodologies published for the separation of neutral and polar acylglycerols using normal-phase (NP) and silica-based reversed-phase (RP) columns (12). Although NP-HPLC is used for the separation of lipid classes, separation of the components of each class is usually accomplished by RP-HPLC. Fatty acids and neutral acylglycerols can be separated with an RP-HPLC method that uses a quaternary stepped mobile phase gradient (13). However, the successful separation of phosphoglycerides requires the inclusion of a modifying salt in the mobile phase. For example, an RP-HPLC method described by Patton *et al.* (14) used 20 mM choline chloride in methanol/water/acetonitrile as the mobile phase. More recently, it was shown that inclusion of ammonium acetate in the mobile phase greatly increased peak sharpness (15,16).

For monitoring the elution of acylglycerols and phosphoglycerides ultraviolet (UV) detection at 205 nm is often used. However, there are two significant disadvantages to the use of a UV detector. First, solvents with significant absorption above 200 nm cannot be used in the mobile phase and, second, the degree of unsaturation in the sample strongly affects detector sensitivity. A detector that does not have these deficiencies is the light-scattering detector (LSD). On the other hand, its use is limited when salts or buffers are used in the mobile phase. Although 0.1% ammonium acetate may be

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Abbreviations: 1,3-DL, 1,3-dilinolein; DO, 1,3-diolein; HPLC, high-performance liquid chromatography; LA, linoleic acid; LSD, light-scattering detector; 1L-2S-PC, 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; LOX, lipoxygenase; NP, normal phase; 1P-2L1-PE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; RP, reversed phase; TL, trilinolein; UV, ultraviolet.

used with an LSD, the addition of choline chloride results in unacceptable baseline noise. It occurred to us that a polymer-based RP column would adsorb the polar functionalities of phosphoglycerides less tightly than a silica-based RP column, alleviating the necessity for the addition of mobile phase salts and buffers. This would be an important advance, as it would allow the utilization of an LSD for the quantitation of all lipids species. Therefore, a polymer-based RP-HPLC method was developed for the analysis of mixtures of free LA, phosphoglycerides and acylglycerols containing linoleate using a mobile phase that was free of salt. This method was utilized to determine the relative rate of oxidation by LOX of these lipid classes.

MATERIALS AND METHODS

Materials. Soybean LOX (lipoxidase, Type 1-B), LA, trilinolein (TL), 1,3-dilinolein (1,3-DL) and 1,3-diolein (DO) were purchased from Sigma (St. Louis, MO). Avanti Polar Lipids, Inc. (Alabaster, AL) supplied 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (1P-2L-PE) and 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (1L-2S-PC). All HPLC solvents (hexane, acetonitrile and methanol) were obtained from Burdick and Jackson (Muskegon, MI). Water was purified to a resistance of 18 M Ω -cm using a Barnstead (Dubuque, IA) NANOpure system. All other reagents were of the highest purity available.

Formation of hydroperoxide. An aliquot of the substrate (6 μ mol linoleate) and 2 μ mol of DO (as internal standard) dissolved in chloroform was placed in a 10-mL Erlenmeyer flask, and the solvent removed under a stream of dry nitrogen. In addition to the substrate, the reaction medium contained 0.2 mg LOX, 0.2 mL 100 mM deoxycholate and 1.8 mL aqueous buffer, consisting of an equal 0.1 M mixture of the buffers AMP, TRICINE, HEPES and MES (respectively, 2-amino-2-methyl-1-propanol hydrochloride, N-tris(hydroxymethyl)-methylglycine, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), and 2-N-morpholino)ethanesulfonic acid). Oxidation was conducted at 15°C with agitation at 250 rpm for an indicated time. Reactions were quenched with 400 μ L 1M citric acid and extracted within 1 h with two 1.5 mL aliquots of chloroform/methanol (2:1, vol/vol). The solvent was removed under a stream of nitrogen, and the sample was redissolved in 0.5 mL of methylene chloride.

HPLC. Liquid chromatographic analysis was performed with a Hewlett-Packard (Palo Alto, CA) Model 1050 HPLC equipped with a gradient program and autosampler. Detection of analytes was by a variable wavelength UV detector, HP 1050 Series, connected in series with a Vorex IIA Universal Evaporative Light-Scattering Detector (ELSD, Rockville, MD) operated at 100°C with nitrogen (40 psi) as the nebulizer gas. All analyses were accomplished with a HEMA-RP C18 10 micron column (250 mm \times 4.6 mm) at programmed flow rate (1 to 1.5 mL/min). Also, the studies included an ADSORBOSPHERE C18 5-micron (250 mm \times 4.6 mm) column. Both columns were obtained from Alltech (Deerfield,

IL). Other types of silica-based columns (CHROMSPHER Si, 5 μ , 200 mm \times 3 mm, and Chrompack Chromsep 5 μ LICHROSORB DIOL, 5 μ , 200 mm \times 3 mm, both supplied by Chrompack, Raritan, NJ) were evaluated but were found to give inferior separations.

Time course and relative rate analysis. A typical reaction time course consisted of seven time points taken at five-minute intervals. The data for each time point was derived from a separately prepared reaction mixture. Each data point represents the mean of at least three injections of 20 μ L of the reaction extract. Oxidations were followed by monitoring with both a UV detector (205 nm) and an LSD. Comparable results were obtained with both detectors. The analysis of the data from the mixtures of substrates to obtain the relative rate of oxidation was accomplished using the computer program developed by Schellenberger *et al.* (11).

RESULTS AND DISCUSSION

HPLC analysis. Preliminary studies on the separation of a mixture of free fatty acid, acylglycerols and phosphoglycerides were made with a silica-based ADSORBOSPHERE RP column. Although several gradient shapes, and combinations of solvents (e.g., isopropanol, acetonitrile, methanol, 1% ammonium acetate in methanol, water) were tried, phosphoglycerides eluted as significantly tailed peaks, and quantitation was not possible. Addition of 20 mM choline chloride to the mobile phase reduced tailing noticeably, as described previously (10,14). However, it was found that the concentration of choline chloride in the mobile phase could be reduced to 1 mM if the column was first equilibrated with 20 mM choline chloride for 1–2 h. Total removal of this modifier resulted in slowly increasing peak tailing of the phosphoglyceride with time. In addition to choline chloride, it was found that the addition of base was needed to obtain completely symmetrical phosphoglyceride peaks. Trimethylamine, ammonium hydroxide and KOH were tested as bases, and the best performance was obtained with 5 mM KOH as a component of the elution system. The best mobile phase gradient that was found is shown in Table 1. Using this gradient, the

TABLE 1
Mobile-Phase Gradient and Flow Rate Program for Separation of Linoleic Acid, 1-Palmitoyl-2-Linoleoyl-*sn*-Glycerol-3-Phosphoethanolamine (1P-2L-PE), 1-Linoleoyl-2-Stearoyl-*sn*-Glycerol-3-Phosphocholine (1L-2S-PC) and Trilinolein on an ADSORBOSPHERE C18 Column^a

Time (min)	Composition of mobile phase ^b			Flow rate (mL/min)
	Eluant A ^c	Eluant B ^d	Eluant C ^e	
0	95	5	0	0.5
4	95	5	0	0.8
14	95	5	0	1.9
16	88	5	7	1.9

^aFrom Alltech (Deerfield, IL). ^bComposition in % (vol/vol). ^cEluant A: 5 mM KOH in methanol. ^dEluant B: 20 mM choline chloride in methanol. ^eEluant C: hexane.

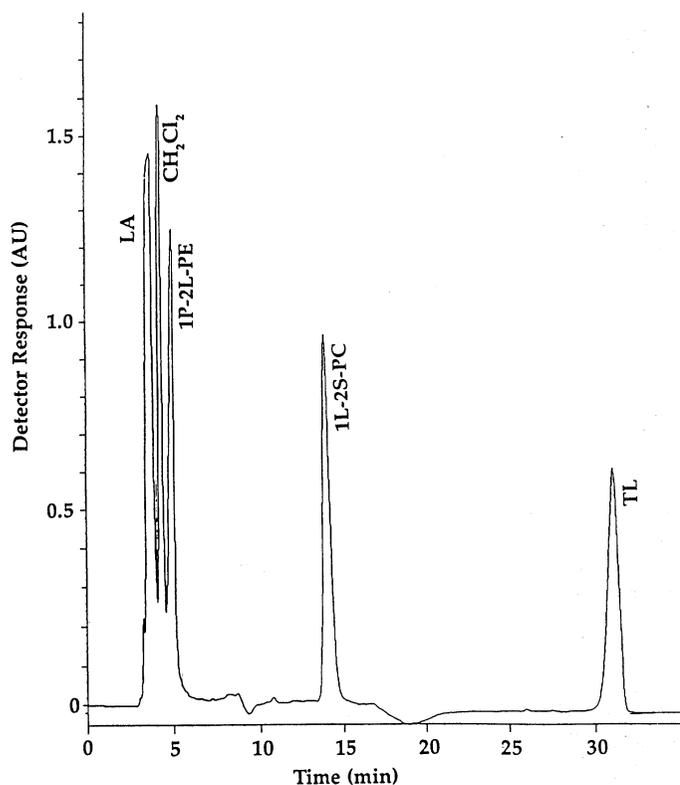


FIG. 1. Elution profile of the injection of 20 μ L of a mixture of linoleic acid (LA), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (1P-2L-PE), 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (1L-2S-PC) and trilinolein (TL) (2 mg/mL in methylene chloride) with silica-based reversed-phase high-performance liquid chromatography using the solvents and modifiers described in Table 1. An Alltech (Deerfield, IL) 5 micron ADSORBOSPHERE C18 column (250 mm \times 4.6 mm) was used, with ultraviolet detection at 205 nm.

separation of LA, 1P-2L-PE, 1L-2S-PC and TL was attempted. The elution profile is shown in Figure 1, and it can be seen that the peaks corresponding to the LA, the injecting solvent, methylene chloride and 1P-2L-PE were not satisfactorily resolved. Due to the presence of an unsatisfactory level of baseline noise in the LSD, only the UV detector was used. The inclusion of KOH in the mobile phase also rapidly cleaves the organic layer from the silicate particles and causes damage to the silica structure of the column, decreasing its resolution and lifetime (17). For these reasons this method was abandoned. Other silica-based columns (CHROMSPHER Si, 5 μ , and LICHROSORB DIOL, 5 μ) were also tested but were found to be unsatisfactory for the separation of this mixture of components.

Because of problems encountered with the silica-based RP column, a polymer-based (hydroxyethyl methacrylate/dimethacrylate copolymer) HEMA-RP C18 column was evaluated. This column is resistant to bases and lacks the interactions associated with the free silanols in the silica-based RP column. After trying different mobile phases, the best separation was achieved with a ternary gradient of water/methanol/acetonitrile, without the need for inclusion of salt, buffer

TABLE 2
Mobile-Phase Gradient and Flow Rate Program for Separation of Linoleic Acid, 1P-2L-PE, 1L-2S-PC and Trilinolein on an HEMA-RP C18 Column^a

Time (min)	Composition of mobile phase ^b			Flow rate (mL/min)
	Eluant A ^c	Eluant B ^d	Eluant C ^e	
0	86	14	0	1.0
5	90	10	0	1.0
8	90	10	0	1.5
10	65	5	30	1.5
13	48	1	51	1.5

^aCompany source and abbreviations as in Table 1. ^bComposition in % (vol/vol). ^cEluant A: methanol. ^dEluant B: water. ^eEluant C: acetonitrile.

or base (Table 2). Figure 2 shows the chromatogram for the separation of the mixture of LA, 1P-2L-PE, 1L-2S-PC, 1,3-DL, DO and TL with the HEMA-RP C18 column. All components, including phosphoglycerides, eluted as sharp peaks. The solvent gradient shown in Table 2 was used for elution and detection was by the LSD.

Comparative kinetic study. A mixture of LA, 1L-2S-PC and TL was prepared in the presence of 10 mM deoxycholate and was subjected to the action of LOX. The concentrations of substrates over time were determined by HPLC using both

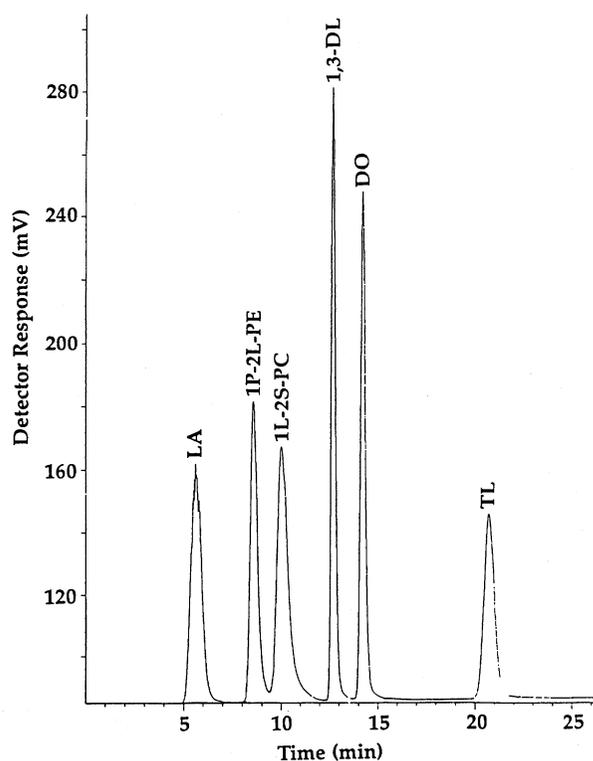


FIG. 2. Elution profile of the injection of 20 μ L of mixture of LA, 1P-2L-PE, 1L-2S-PC, 1,3-dilinolein (1,3-DL), 1,3-diolein (DO) and TL (2 mg/mL in methylene chloride) with polymer-based reversed-phase high-performance liquid chromatography using the solvents described in Table 2. An Alltech 10 micron HEMA-RP C18 column (250 mm \times 4.6 mm) was used, with light-scattering detection. Abbreviations and company source as in Figure 1.

the LSD and the UV detector. However, at the longer time points the concentration of LA became very low, and only the UV detector was sensitive enough to measure the remaining LA. Figure 3 shows the chromatogram of the substrates and hydroperoxides formed from a mixture of LA, 1L-2S-PC and TL after 30 min of reaction. Peaks B, D and G correspond to LA, 1L-2S-PC and TL, respectively. Peaks A, C and F are the corresponding hydroperoxide products. Peak E is the internal standard (DO). The assignment of each hydroperoxide was made by oxidation of each substrate separately. Figure 4 shows a typical time course of LOX-catalyzed oxidation of LA, 1L-2S-PC and TL. The relative rate of oxidation of the multiple substrates was calculated by the method of Schellenberger *et al.* (11), and the results are presented in Table 3. As expected, LA was oxidized at the fastest rate, and the rates of oxidation of 1L-2S-PC and TL were only 10% of that of LA. Control experiments showed that nonenzymatic oxidation did not occur over the time-span of the experiment.

The competitive oxidation of LA and 1,3-DL by LOX was also monitored by HPLC. Surprisingly, 1,3-DL was oxidized at a rate that was 40% as fast as LA (Table 3). The relatively high rate of oxidation of 1,3-DL as compared to TL and 1L-2S-PC suggests that steric factors strongly influenced the ability of LOX to promote hydroperoxide formation. The oxida-

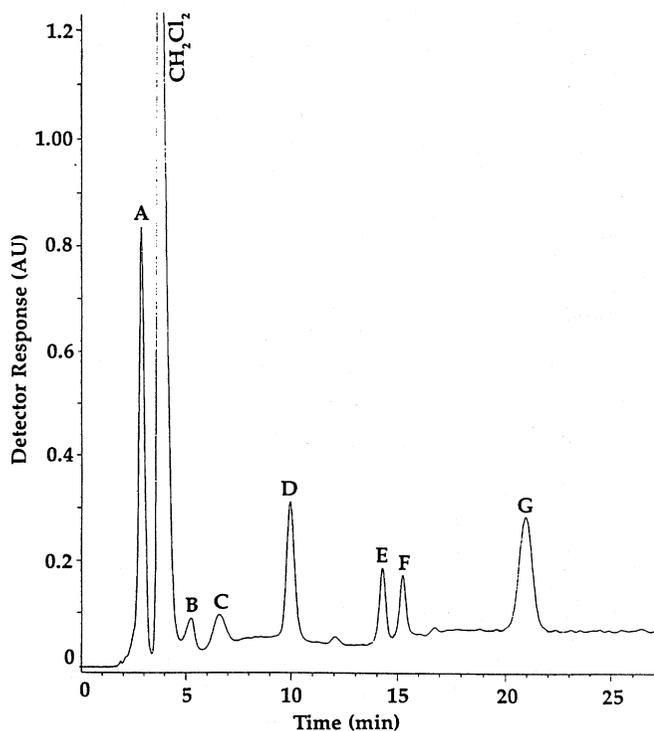


FIG. 3. Elution profile of a mixture of LA, 1L-2S-PC and TL after 30 min of incubation with lipoyxygenase, with polymer-based reversed-phase high-performance liquid chromatography using the solvents described in Table 2. An Alltech 10-micron HEMA-RP C18 column (250 mm \times 4.6 mm) was used, with ultraviolet detection at 205 nm. A, peroxide of LA (B); C, peroxide of 1L-2S-PC (D); E, DO (internal standard); F, peroxide of TL (G). Abbreviations and company source as in Figures 1 and 2.

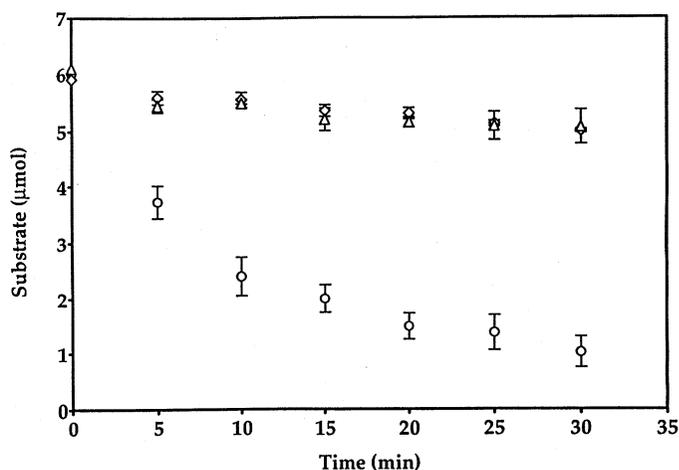


FIG. 4. Time course of the oxidation by lipoyxygenase of a mixture of LA (\circ); TL (\diamond) and 1L-2S-PC (\triangle), as determined by reversed-phase high-performance liquid chromatography. The chromatographic conditions are described in Table 2. Abbreviations as in Figure 1.

TABLE 3
Relative Rate of Oxidation of Linoleic Acid (LA), 1L-2S-PC, Trilinolein (TL) and 1,3-DL by Lipoyxygenase^a

Substrates	Relative rate (%) ^b
LA	100
1L-2S-PC	11 \pm 1
TL	10 \pm 3
LA	100
1,3-DL	36 \pm 2

^aEach determination consisted of seven samples containing 6 μ mol linoleate for each substrate, 10 mM deoxycholate, and 0.2 mg lipoyxygenase at pH 8 and 15°C. Samples were analyzed every 5 min from time 0 to 30. Abbreviations as in Table 1.

^bThe data are the means \pm SD (n = 6).

tion of 1,3-DL by LOX led to the formation of mono- and dihydroperoxides as major products, with small amounts of other unidentified products as determined by HPLC.

In conclusion, the use of the HPLC with a polymer-based column allowed for the separation and quantitation of a mixture of free fatty acid, acylglycerols and phosphoglycerides without the need of modifiers in the mobile phase. Detection by both LSD and UV can be made. The utility of this HPLC method was demonstrated using chemically pure substrates. However, this method should be useful for analyzing the extent of oxidation in many fats and oils.

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