

Chapter 11

Immobilization of Soybean Lipoxygenase and Promotion of Fatty Acid and Ester Oxidation

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Introduction

While other chapters in this monograph have discussed the structural and mechanistic features of lipoxygenase and the physiological impact of its metabolites, this chapter consists of a discussion of work on the immobilization of lipoxygenase from soybeans and its use in oxidizing fatty acid esters. Others have demonstrated that hydroperoxides are useful chiral synthons for further chemical modification (1-5). The study of enzymes that use fatty acid hydroperoxides as their substrates is still in a developing stage. As our knowledge of these enzymes grows, ways of using them for practical syntheses will be devised, and the richness of synthetically useful chemical structures derived from hydroperoxides will expand manifold. As indicated in part by the large number of free and immobilized lipases that are currently available from commercial sources, the age of chemical synthesis with enzymes has arrived. Other chapters in this monograph have alluded to diseases that are linked to the action of lipoxygenase, and certainly the food industry views lipoxygenase activity as a potential problem in product quality (6). However, this chapter emphasizes the potential utility of a much-maligned enzyme in chemical synthesis.

Practical Problems Associated with the Use of Free Lipoxygenase

Free soybean lipoxygenase is unstable in solution, losing most of its activity within a day. This instability problem is exacerbated when high oxygen levels are achieved by bubbling oxygen into an aqueous buffer containing a fatty acid salt. Fatty acid salts have detergent properties, and much foam is formed. The resulting surface tension in the medium surrounding each bubble has a propensity to promote enzyme denaturation. Existing laboratory procedures for promoting high conversion to hydroperoxide require the repeated addition of small amounts of lipoxygenase to compensate for rapid enzyme inactivation. To circumvent this problem, syntheses have been con-

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ducted in high-pressure bombs (7), in the presence of antifoam (3), and in media containing high levels of organic solvents since oxygen is much more soluble in nonpolar organic solvents than in aqueous buffers (8–10). Also it has been reported that the presence of an organic solvent is beneficial because it reduces substrate aggregation (9). If the source of lipoxygenase is inexpensive enough to be compensated for by the increased value of the resulting product, then the use of free lipoxygenase is acceptable. However, it has been recognized that enzyme recycling by binding it to a matrix could result in added economy (11). In addition, immobilized enzymes are sometimes more stable than their free counterparts.

Immobilization of Lipoxygenase

Published literature on lipoxygenase immobilization is very limited. Lipoxygenase was immobilized on cyanogen bromide-activated agarose (12). The activity of the immobilized preparation was nearly identical to that of free lipoxygenase. It retained activity at room temperature for at least 1 month. A functional bioreactor was prepared using lipoxygenase immobilized on cyanogen bromide-activated Sepharose (13). The flow rate and the concentration of the substrate, linoleic acid (LA), were fixed, and the amount of immobilized material was adjusted to determine the amount needed to most efficiently convert LA to its corresponding hydroperoxide. The conversion efficiency, in terms of product formed per unit time, could be further increased by increasing the flow rate up to a point, but if the flow rate was too high, the yield of oxygenated product was reduced.

Experiments were performed using lipoxygenase adsorbed through hydrophobic interactions, covalently bound by cyanogen bromide and physically adsorbed, then cross-linked by glutaraldehyde (14). Lipoxygenase adsorbed onto wetted porous glass or on porous silica showed low catalytic activity in *n*-decane, whereas lipoxygenase adsorbed onto the amphiphilic gels octyl- and phenyl Sepharose exhibited high activity in *n*-decane. All of the immobilized lipoxygenase preparations, adsorbed and covalently bound, had half-lives of several hours. Soybean lipoxygenase-1 (SBL-1) was covalently immobilized on oxirane acrylic beads (15). Very high yields of the LA hydroperoxide were obtained when the beads were used in an oxygenated, aqueous buffer. The beads could be reused, but information on the stability of this lipoxygenase preparation was not presented. Very recent work has investigated the adsorption of plant lipoxygenases onto talc (16,17). Lipoxygenases isolated from *Solanum tuberosum* tuber and *Lupinus albus*, *Cicer arietinum*, and *Pisum sativum* seeds were utilized. When stored at 4°C the adsorbed lipoxygenases from all sources showed greatly enhanced stability compared to free controls. The preparation derived from *S. tuberosum* was tested for reusability. Its activity dropped by only 34% after 10 uses.

Our laboratory has investigated the immobilization of soybean lipoxygenase (Sigma, Lipoxidase, Type 1-B) on a commercially available carbonyldi-imidazole-activated support (Pierce, ReactiGel® 6X [18]). The urethane linkage formed when a protein is bound to this support is about 20 times more stable than the *N*-substituted

isourea linkage formed during protein immobilization onto cyanogen bromide-activated matrices (19). The immobilized preparation of lipoxygenase was extremely stable at 5°C, losing only 5% of its activity in 6 months. At 15°C, the immobilized lipoxygenase had reduced stability, but as shown in Figure 11.1, its stability was still much greater than that of free lipoxygenase. The half-life of free lipoxygenase was approximately 7 hours. The half-life of immobilized lipoxygenase was estimated to be 75 hours by replotting the activity data on a logarithmic scale and using a linear least-squares regression fit (20).

Catalytic Properties of Immobilized Lipoxygenase

The enzymatic activity of lipoxygenase immobilized on ReactiGel was examined at 15°C in mixtures containing organic solvent and aqueous buffer in which air was the sole source of oxygen (21). The amount of hydroperoxide, hydroperoxyoctadecadienoic acid (HPOD), formed in water-saturated *n*-octane from LA in 3 hours as the amount of aqueous buffer increased is shown in Figure 11.2. Although some HPOD formed even when no aqueous buffer was added, having 35% v/v aqueous buffer resulted in a threefold increase in the amount of HPOD.

The pH of the aqueous buffer also had a strong influence on the amount of HPOD formed (Fig. 11.3). As in all aqueous media, the amount of HPOD formed was maximized at pH 9–9.5. Hydroperoxyoctadecadienoic acid formation rapidly decreased as the pH was lowered. At pH values higher than 9.5, a more gradual decrease in HPOD formation occurred.

Figure 11.4 shows the influence of a variety of organic solvents on HPOD formation. The most HPOD was formed in 1,1,2-trichlorotrifluoroethane. Approximately equal amounts of HPOD were formed when the solvents hexane, heptane, octane, and 2,2,4-trimethylpentane were used. Still lower levels of HPOD were formed in toluene and cyclohexane; the lowest detectable levels of HPOD were found in reaction mixtures that contained diethyl ether and di-isopropyl ether. No HPOD formation was detected with the solvents 2-butanone or 2-octanone. These results are consistent with other work showing that polar organic solvents generally are detrimental to enzymic activity (22).

With some enzymes, the solvent can have a profound effect upon regioselectivity (23). As has been discussed previously, a fatty acid substrate in media containing both aqueous and organic components would have a propensity to be oriented in such a way that its polar head group is found in or is oriented toward the aqueous fraction where lipoxygenase resides. This might result in a reversal of the regiospecificity of lipoxygenase, such that the Δ -9 carbon receives the hydroperoxide functionality, rather than the Δ -13 carbon as is observed in all aqueous media. Recently this possibility was tested in reverse micelles containing Aerosol OT [sodium *bis*(2-ethylhexyl) sulfosuccinate] and octane (24). The 13-hydroperoxide isomer was the predominant product formed, indicating that the orientation of the substrate on lipoxygenase was not influenced by its orientation in the bulk reaction medium. We have also examined

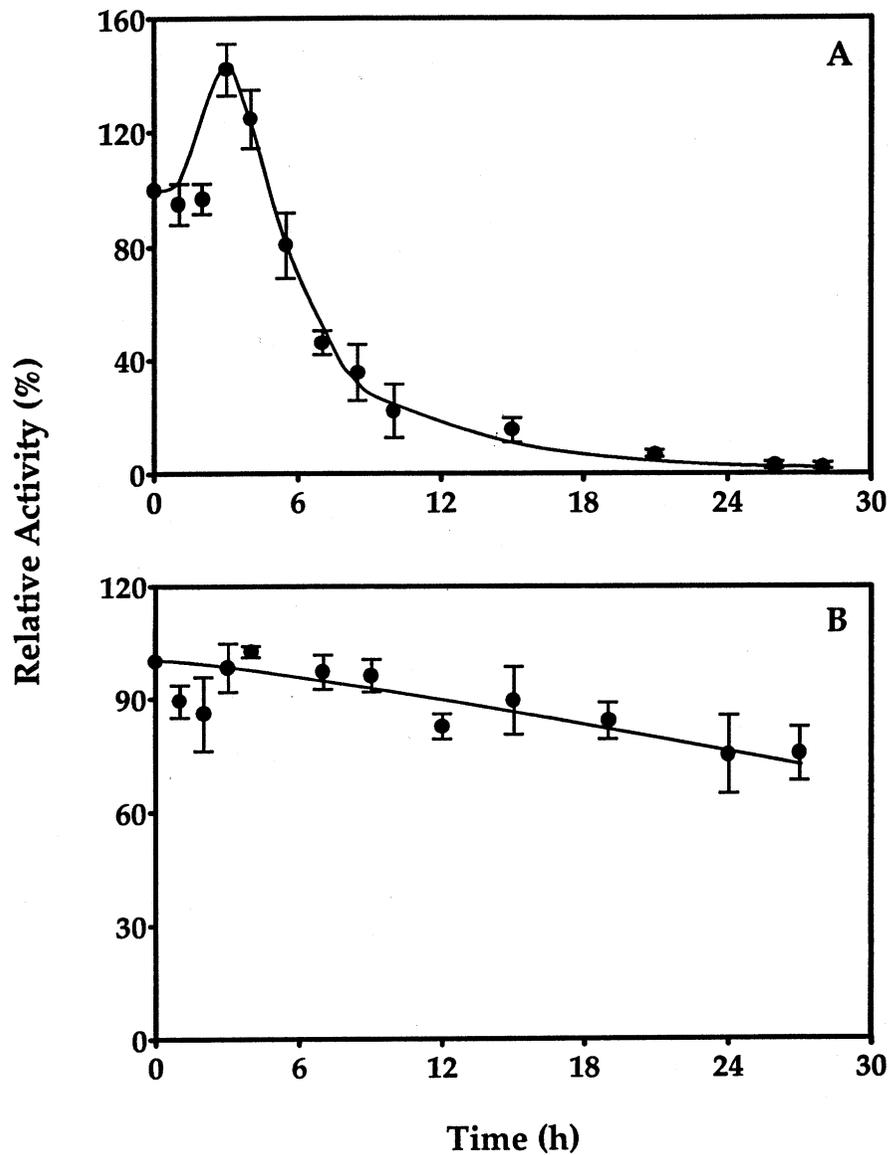


Figure 11.1. Stability of unbound lipoxigenase *a*) and immobilized lipoxigenase *b*) at 15°C. Lipoxigenase (2 mg of powder containing 1 mg of protein) or immobilized lipoxigenase (0.194 g of gel containing 1 mg of protein) was dispersed in 14.5 mL of Tricine buffer, pH 9.0. At the indicated time, 40 mg of LA in 100 μ L of ethanol was added. The reaction was allowed to proceed for 15 min. Results are means \pm SEM for four determinations.

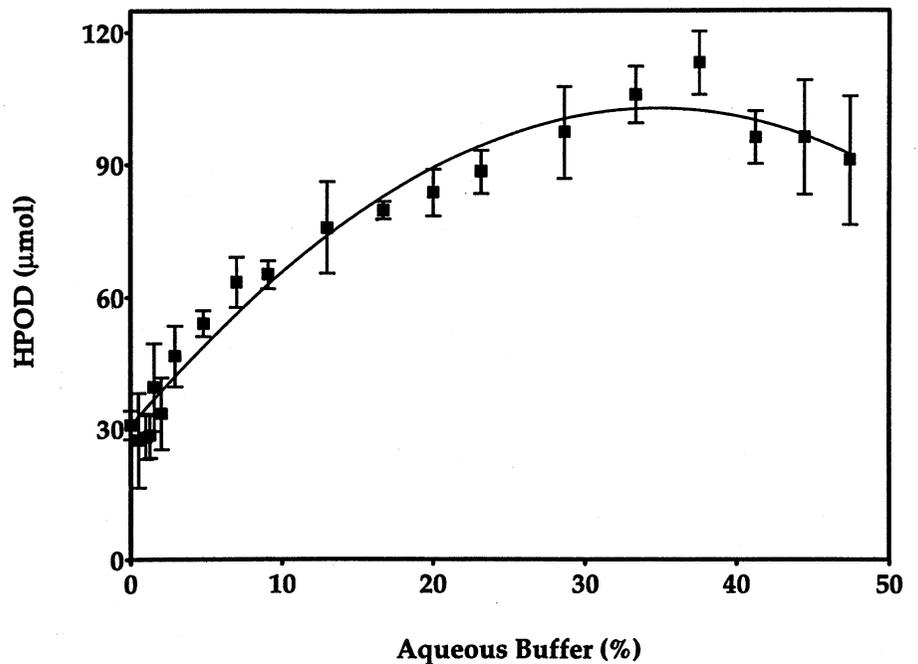


Figure 11.2. Influence of the amount of 0.2 M aqueous borate buffer, pH 9.0, on HPOD formation catalyzed by immobilized lipoxygenase. In addition to the buffer, each assay contained 0.75 g immobilized lipoxygenase containing 3.0 mg bound protein, 40 mg LA, and 15 mL water-saturated *n*-octane. Assays were conducted for 3 h at 15°C. The percentage buffer values were calculated: mL buffer/(mL buffer + mL octane) × 100. The data are the means ± SEM of 10 determinations.

the specificity of immobilized lipoxygenase in media containing both aqueous buffer and hexane (21). Again the 13-hydroperoxide isomer was found to be the predominant product formed.

Action of Lipoxygenase upon Linoleate Esters

Lipoxygenase from different sources and lipoxygenase isozymes from the same source exhibit different degrees of specificity depending on whether the carboxylic acid moiety is free or esterified. The high-pH form of lipoxygenase from soybean, SBL-1, is highly specific for free fatty acid under most conditions, while lipoxygenases from other sources are able to oxidize phospholipids containing the esters of polyunsaturated fatty acids. It has been particularly well documented that lipoxygenases from mammalian sources can oxidize phospholipids (25,26), as well as membranes and lipoproteins primarily containing esterified fatty acids (27–29). However,

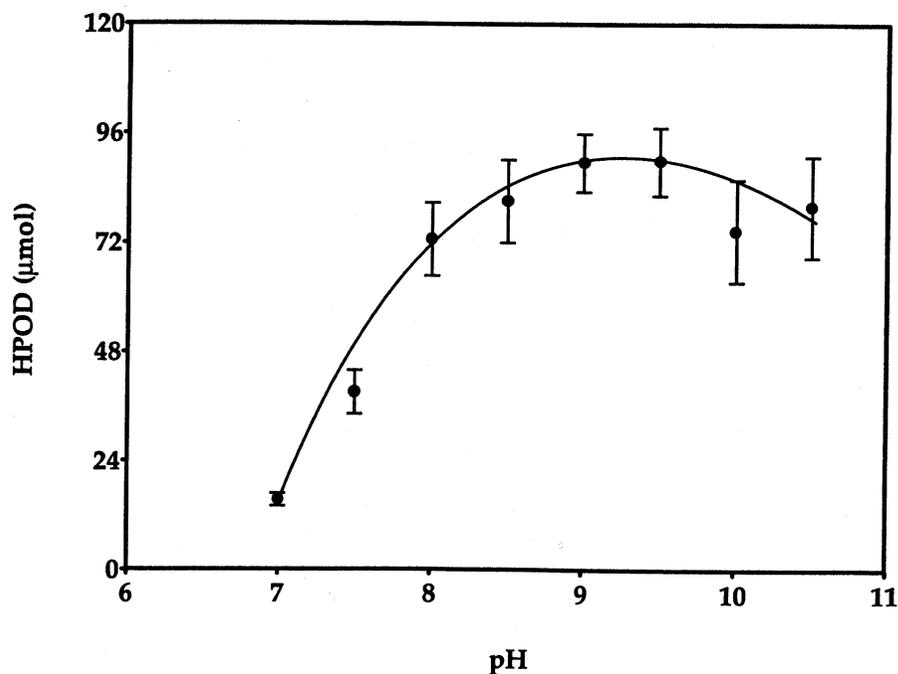


Figure 11.3. Influence of buffer pH on HPOD formation catalyzed by immobilized lipoxygenase. The assays contained 6 mL of buffer containing a mixture of 0.1 M Hepes, Tricine, and 2-amino-2methyl-1-propanol, hydrochloride, 15 mL water-saturated *n*-octane, 0.67 g immobilized lipoxygenase containing 3 mg bound protein, and 40 mg LA. The assays were conducted for 3 h at 15°C. The data are the means \pm SEM of six determinations.

some plant lipoxygenase isozymes can oxidize membrane fractions and purified phospholipids (30–32).

Early work on SBL-1 showed that in the presence of Tween 20, free LA was rapidly oxidized, but the LA methyl ester and trilinolein were poor substrates for lipoxygenase (33). It was concluded that lipoxygenase requires the prior action of lipase or phospholipase to release free fatty acid *in vivo*. Modification of this view was required when it was discovered that soybean lipoxygenase had significant activity with phosphatidylcholine in the presence of bile salt, deoxycholate, cholate, or taurocholate (34). No oxidation occurred in Tween 20, Triton X-100, Tween 80, sodium dodecyl sulfate (SDS), and octyl glucoside. The detergent 3-16 Zwittergent promoted very slow oxidation. Except for SDS, lipoxygenase inactivation by the detergents was eliminated as a reason for the lack of support of oxidation. In addition, it was shown that release of free fatty acid from phosphatidylcholine did not occur prior to oxidation.

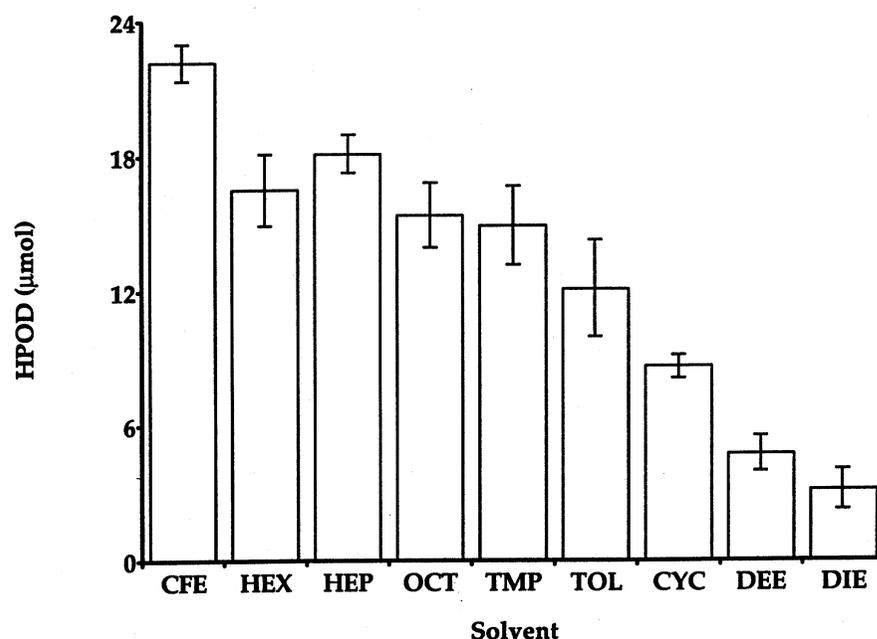


Figure 11.4. Influence of organic solvent on HPOD formation catalyzed by immobilized lipoxygenase. Each assay contained 15 mL water-saturated organic solvent, 6 mL 0.2 M borate buffer, pH 9.0, 0.194 g immobilized lipoxygenase preparation containing 1 mg bound protein, and 40 mg LA. The assays were conducted for 1 h at 15°C. Results are means \pm SEM of three repetitions.

Abbreviations: 1,1,2-trichlorotrifluoroethane, CFE; hexane, HEX; heptane, HEP; octane, OCT; 2,2,4-trimethylpentane, TMP; toluene, TOL; cyclohexane, CYC; diethyl ether, DEE; di-isopropyl ether, DIE.

This study was extended by an examination of the structure of the oxidized arachidonyl and linoleoyl moieties in phosphatidylcholine that had been exposed to soybean lipoxygenase in the presence of deoxycholate (35). It was found that regio- and stereospecific hydroperoxide formation took place. Also, when oxidation was conducted in ^{18}O -labeled water, there was no incorporation of ^{18}O in the product, eliminating the possibility of hydrolysis and reesterification during oxidation.

These studies show that phosphatidylcholine is a true substrate for lipoxygenase. Phosphatidylethanolamine was also shown to be a substrate for lipoxygenase (36). When oxidation reactions conducted with soybean-derived phosphatidylethanolamine and phosphatidylcholine were subjected to fractionation by HPLC, it was shown that the oxidized species contained the following fatty acid pairs: 18:3/18:2, 18:2/18:2, and 16:0/18:2.

A single report has shown that lipoxygenase can oxidize fatty acid amides (37). Arachidonylethanolamide was oxidized by porcine leukocyte 12-lipoxygenase and rabbit reticulocyte and soybean 15-lipoxygenases. Human platelet 12-lipoxygenase oxidized the amide only very slowly, while porcine leukocyte 5-lipoxygenase was totally inactive.

Recently the action of soybean lipoxygenase upon neutral glycerides and the LA methyl ester has been investigated (38; Piazza et al., unpublished results). These studies were performed with free lipoxygenase. Presumably the advantages of using immobilized lipoxygenase on LA would also apply with esterified substrates, but this has not yet been tested.

Table 11.1 shows the amounts of oxidation products formed by the action of SBL-1 on several neutral esters in 15-minute assays. Without surfactant, only LA was oxidized at a rapid rate. In Tween 20, LA and monolinolein were oxidized rapidly. Linoleic acid, methyl linoleate, and monolinolein were oxidized rapidly in the presence of deoxycholate. The rate of dilinolein oxidation was about one-half the rate of LA oxidation. Trilinolein was oxidized at a very slow rate. Methyl linoleate and trilinolein have approximately equal solubility in the assay buffer, and thus their highly different rates of oxidation by lipoxygenase must be due to steric constraints in the active site.

The pH profiles of the action of soluble soybean lipoxygenase upon trilinolein and 1,3-dilinolein are shown in Figure 11.5. Both profiles are similar with the optimal pH for oxidation being approximately 8.0. The oxidation rate diminishes at both higher and lower pH values, although action against trilinolein decreases more slowly as the pH is reduced.

TABLE 11.1 Relative Amounts of Oxidized Linoleate Formed by Lipoxygenase in 15 min.

Substrate	Relative Amount of Oxidation ^a		
	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		

^aAssays were conducted in 10 mL Erlenmeyer flasks, each containing substrate (6 μmol linoleoyl residues), 25 μg lipoxygenase, 0.2 mL surfactant or water, and 1.8 mL aqueous buffer, consisting of an equal 0.1 M mixture 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. The amount of LA oxidized in the presence of deoxycholate was 0.986 μmol (16.4% available LA). Results are means ± SEM for three repetitions.

^bThe deoxycholate concentration was 10 mM.

^cThe Tween 20 concentration was 0.25% (v/v). Source: Christopher, Pistorius, and Axelrod (33).

^dResults were not significantly different from zero.

^eData from Piazza and Nuñez (38).

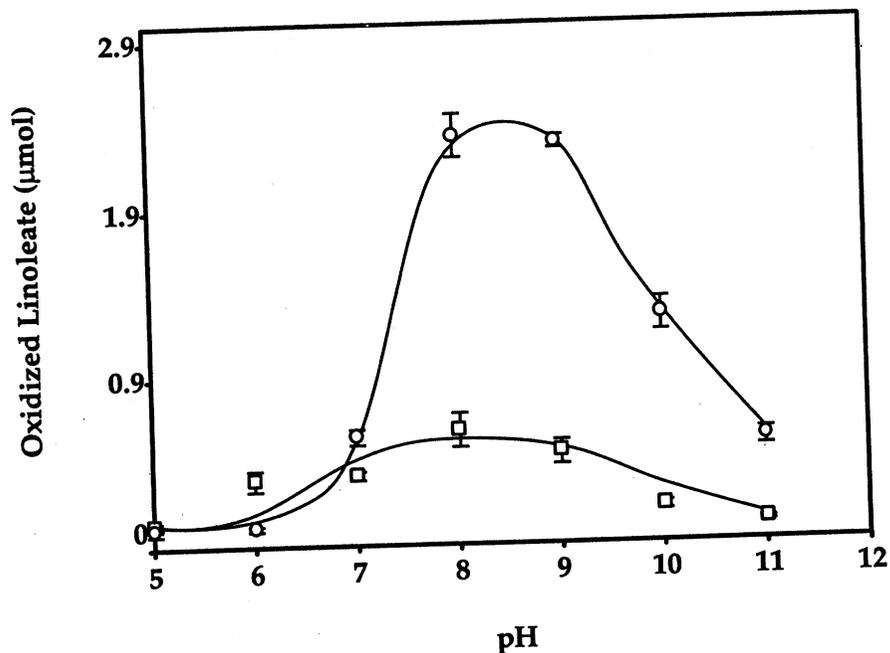


Figure 11.5. Influence of pH on the oxidation of 2 μM trilinolein (\square) and 3 μM 1,3-dilinolein (\circ) by free lipoxygenase in the presence of deoxycholate. Assays were conducted as described in Piazza and Nuñez (38). Results are means \pm SEM of three repetitions.

As is clear from Figure 11.5 and Table 11.1, the overall amount of trilinolein oxidized is lower than that of dilinolein within the time period studied. To achieve a better understanding of the potential of lipoxygenase to oxidize these two compounds, complete-reaction-time courses were followed. These showed that only 15% of the available linoleoyl residues were oxidized in trilinolein, and increasing the amount of lipoxygenase did not increase the extent of oxidation. Although direct evidence is lacking, this result may be due to inhibition by the oxidation product. In contrast to the results obtained with trilinolein, 67% of the available linoleate in dilinolein was converted to hydroperoxide by lipoxygenase. When dilinolein oxidation was monitored using HPLC, all of the dilinolein was eventually oxidized, but other unidentified polar materials were formed in addition to mono- and dihydroperoxides. Although exact yields are not available as of this writing, it is clear that both methyl linoleate and monolinolein can also be oxidized by soybean lipoxygenase to a high degree.

Conclusions

In conclusion, soybean lipoxygenase can be immobilized to provide an efficient catalyst for the regio- and stereospecific introduction of hydroperoxide into fatty acids. Recent advances have demonstrated that oxidations can be conducted in reverse micelles, in organic solvent–aqueous mixtures, and that neutral and charged fatty acid esters can be substrates for soybean lipoxygenase provided that proper reaction conditions are utilized. These results indicate that soybean lipoxygenase has the potential to act upon a wide variety of structurally disparate molecules and will eventually find a role in the arsenal of the synthetic chemist.

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