ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 354, No. 1, June 1, pp. 18–23, 1998
Article No. BB980673

Oxidation of Dilinoleoyl Phosphatidylcholine by Lipoxygenase 1 from Soybeans

M. Pérez-Gilabert, G. A. Veldink,[1] and J. F. G. Vliegenthart
Bijvoet Center for Biomolecular Research, Department of Bio-organic Chemistry, Utrecht University, NL-3584 CH Utrecht, The Netherlands

Received December 31, 1997, and in revised form March 16, 1998

Soybean lipoxygenase-1 is able to oxidize dilinoleoyl phosphatidylcholine at pH 7.5 and 10. The reaction could be followed spectrophotometrically from the increase of the absorbance at 234 nm. An intermediate product and a final product were detected. In the intermediate product only one of the linoleoyl chains (either *sn1* or *sn2*) was oxidized. In the final product, both linoleic acid units were converted into hydroperoxides. Apparently, oxidation of one of the linoleoyl chains leads to a disruption of the structure of the mixed bilayer disk, making the remaining fatty acid unit more accessible to the action of the enzyme. The specificity of lipoxygenase-1 when acting on phospholipids is not affected by pH. The exclusive production of 13-hydroperoxyoctadecadienoic acid derivatives of dilinoleoyl phosphatidylcholine at pH 7.5 and 10 may result from the blockage of the carboxylic end of the fatty acid.

Key Words: **deoxycholate; GC/MS; dihydroperoxy-linoleoyl; lysophosphatidylcholine; phosphatidylcholine; phospholipids; SP-HPLC.**

Lipoxygenases are nonheme iron-containing enzymes that use molecular oxygen in the dioxygenation of a fatty acid containing one or more 1,4-*Z*,*Z*-pentadiene systems. These enzymes are ubiquitously distributed in animals and plants and have a key function in the formation of biologically active substances (1, 2). The precursors of these compounds are free fatty acids liberated from membrane lipids via (phospholipase-catalyzed reactions (1–3). Although some lipoxygenase isoenzymes can oxidize certain triglyceride lipids, it is generally acknowledged that free polyunsaturated fatty acids are the preferred substrates. The direct action of mammalian LOXs[2] on phospholipids and biomembranes (4–7) suggests a role of LOXs in processes such as maturation of reticulocytes, which implies a change in the structure of the membrane (8, 9). In plants, pure enantiomers of hydroxy fatty acids have been detected in the storage lipids of cucumber, soybean, tobacco, and rape (10). Kondo *et al.* (11) observed an increase in the activity of LOX toward phospholipids after treatment of soybean seedlings with fungal elicitors. Eskola and Laakso (12) showed that, in the presence of deoxycholate, soybean lipoxygenase-1 catalyzes the dioxygenation of polyunsaturated fatty acids esterified in phosphatidylcholine. The reaction was found to depend on the solubilization of the phospholipid with bile salts. Brash *et al.* (5) analyzed the products of this reaction and showed that arachidonyl and linoleoyl moieties in the phosphatidylcholine were converted exclusively into the 15(*S*)-hydroperoxyeicosatetraonate and 13(*S*)-hydroperoxyoctadecadienoate analogs, respectively. These results provided concrete evidence that fatty acids esterified in phospholipids can be subjected to highly specific oxygenation by lipoxygenase. In this paper, the ability of soybean LOX to oxidize a dilinoleoyl phospholipid has been studied.

MATERIALS AND METHODS

Materials. Dilinoleoyl phosphatidylcholine (DL-PC) (99%) and sodium borohydride were purchased from Sigma (Bornem, Belgium). Boric acid and sodium dihydrogen phosphate monohydrate were from Merck (Darmstadt, Germany). Phospholipase A_2 from *Naya naya* venom was from Fluka AG (Buchs, Switzerland).

[1] To whom correspondence should be addressed. Fax: +31-30-254-0980.

[2] Abbreviations used: DL-PC, dilinoleoyl phosphatidylcholine; HPOD, hydroperoxyoctadecadienoic acid; HOD, hydroxyocta- decadienoic acid; LOX, lipoxygenase; P1, monohydroperoxylinoleoyl phosphatidylcholine; P2, dihydroperoxylinoleoyl phosphatidylcholine; PC, phosphatidylcholine; PLA_2, phospholipase A_2; SP-HPLC, straight-phase HPLC.

0003-9861/98 $25.00

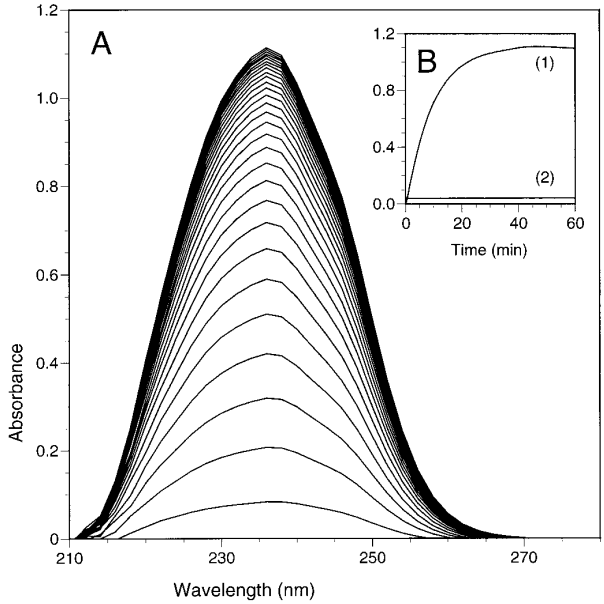


FIG. 1. Difference spectra (A) and progress curve (B, trace 1) for the oxidation of DL-PC by soybean LOX-1 in the standard reaction medium. The final volume is 1 ml; cycle time is 1 min. A control without enzyme is represented in B, trace 2.

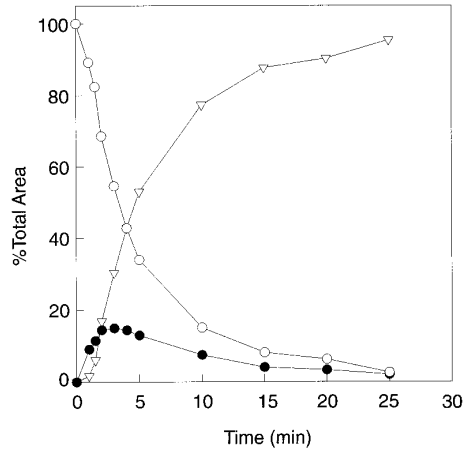


FIG. 2. Relative changes of DL-PC (○), P1 (●), and P2 (▽) during the incubation of DL-PC with LOX-1 in the standard reaction medium. At different times aliquots were taken and the products extracted and analyzed by SP-HPLC. 100% = area (DL-PC + P1 + P2).

1,1,1,3,3,3-Hexamethyldisilazane, trimethylchlorosilane, pyridine, and 0.5 M sodium methoxide were obtained from Aldrich (Milwaukee, WI). Octadecyl solid-phase extraction columns (1 ml, 50 mg) were purchased from Waters (Milford, MA). Sodium deoxycholate was from Janssen Chimica (Geel, Belgium). Chloroform, 2-propanol (Merck, Darmstadt, Germany), methanol (Biosolve, Valkenswaard, The Netherlands), and *n*-hexane (Rathburn Chemicals, Walkerburn, UK) were all of HPLC grade.

Substrate preparation. Aqueous phosphatidylcholine substrates were prepared by drying aliquots of the phosphatidylcholine stock solution in chloroform under a stream of N_2; the film obtained was rapidly dispersed in 10 mM deoxycholate dissolved in 0.2 M sodium borate (pH 10) or 0.1 M sodium phosphate (pH 7.5) buffer. The resulting substrate solution was allowed to equilibrate for 10 min at 25°C (12).

Enzymatic activity. Lipoxygenase-1 from soybeans was purified according to the method of Finazzi Agrò *et al.* (13) and had a specific activity of 20 units per milligram of protein. One unit is defined as the amount of enzyme that gives rise to the formation of 1 μmol of conjugated diene per minute, using linoleic acid as a substrate in 0.1 M borate buffer, pH 9.0. The activity was followed spectrophotometrically in a HP8452A diode array as the increase in absorbance at 234 nm. The standard reaction medium contained 25 μM DL-PC, 1.5 units of lipoxygenase-1, and 10 mM sodium deoxycholate in 0.2 M borate buffer, pH 10. A control without enzyme was also carried out. To compare the relative rates of oxidation for linoleic acid and DL-PC, the oxidation of the free fatty acid was measured in the standard reaction medium. The effect of 13-HPOD and P1 in the oxidation rate of DL-PC was also studied.

TABLE I

Effect of Different Compounds on the Oxidation of DL-PC by Soybean LOX-1

Substrate	Relative activity[a] (%)
25 μM DL-PC	100
25 μM P1	97 ± 6
25 μM DL-PC + 25 μM 13-HPOD	73 ± 2
25 μM DL-PC + 25 μM P1	243 ± 7
25 μM linoleic acid	433 ± 12

Note. In all cases the reaction medium contained 10 mM deoxycholate dissolved in 0.2 M sodium borate (pH 10) and 1.5 enzymatic units of LOX-1.

[a] Mean ± SE, n = 3 measurements.

Isolation of the reaction products. The products of the reaction were extracted according to the procedure of Bligh and Dyer (14) or by using 50 mg octadecyl solid-phase extraction columns; in the latter case, the samples were applied directly in the buffer, the column was washed with water, and the phospholipids were eluted with methanol. The analysis of the reaction products was performed using SP-HPLC on a CP-Sphere Si column (Chrompack, 5-μm particles, 4.6 × 250 mm) using a HP1040A diode array UV detector and a HP7994A analytical workstation for data processing. Products were eluted isocratically with *n*-hexane/2-propanol/H_2O (4:6:1 v/v/v) at a flow rate of 1.0 ml/min and detected at 234 nm. In this system, PC eluted before its oxidation products and was detected at 205 nm.

Derivatization and GC/MS. The esterified lipids were dissolved in methanol and reduced with sodium borohydride at 0°C under N_2. Water was added and the mixture was acidified with 3 M HCl to pH 4. The products were extracted with a octadecyl solid-phase extraction column and eluted with methanol. Transesterification was accomplished by dissolving the phospholipid in 50 μl methanol and adding 50 μl 0.5 M methanolic sodium methoxide. The reaction was allowed to proceed for 10 min at room temperature, 325 μl 0.1 N HCl was added, and the solution was immediately extracted with 2 vol of *n*-hexane. The organic phase was washed once with an equal volume of water and evaporated to dryness under a N_2 stream. The residue was silylated by treatment with a mixture of pyridine/hexamethylsilazane/trimethylchlorosilazane (5:1:1 v/v/v) for 30 min. The resulting compounds were analyzed on a mass spectrometer (Fisons Instruments MD 800 Mass Lab spectrometer, GC 8000 Series Interscience), equipped with a DB-1 fused-silica capillary column (30 m × 0.32 mm, J & W Scientific): temperature program, 2 min isothermal

at 140°C, 140–280°C (6°C/min), 2 min isothermal at 280°C; runtime, 28 min. All mass spectra were recorded under electron impact with an ionization energy of 70 eV.

Phospholipase A_2 assay. The reaction medium contained 100 μl 1 M Tris/HCl, pH 8.5, 100 μl 0.1 M $CaCl_2$, and 175 μl H_2O. P1 (0.05 μmol) was dried under a stream of N_2 and dissolved in 250 μl ether and added to the reaction medium (15). The reaction was started with the addition of 5 μl of 1 mg/ml solution PLA_2. The mixture was incubated for 5 min at room temperature under constant stirring. The ether was evaporated, water was added, and the products were eluted from a C18 column with methanol. Lyso-PC and lyso-PC hydroperoxides were detected at 205 and 234 nm, respectively.

RESULTS

In Fig. 1 repetitive scans (A) and the progress curve at 234 nm (B, trace 1) are shown for the oxidation of DL-PC by soybean LOX-1 in borate buffer, pH 10, containing 10 mM deoxycholate. The oxidation of DL-PC does not take place in the absence of enzyme (Fig. 1B, trace 2). Under these experimental conditions the oxidation rate is 23% of the one obtained using linoleic acid as a substrate. The reaction is stimulated by the addition of P1 to the reaction medium and partially inhibited by 13-HPOD (Table I).

The products of the reaction were extracted by the method of Bligh and Dyer (14) and analyzed by SP-HPLC. This analysis shows that the oxidation of DL-PC by soybean LOX-1 in the presence of deoxycholate gave rise to two products absorbing at 234 nm that we designate P1 (retention time 16 min) and P2 (retention time 21 min). In Fig. 2 the change with time in the relative amounts of the substrate (DL-PC) and of the two products P1 and P2 is shown. During the initial 5 min, the amount of DL-PC dropped rapidly. P1 started to decline after reaching a maximum at 2.5 min. After a lag, P2 continuously increased until it leveled off at the end of the reaction. When the incubation is carried out in the presence of 5 μM P1, the lag phase in P2 formation is abolished. No significant difference with respect to the control (Fig. 2) is observed when 5 μM 13-HPOD is added to the incubation medium (data not shown). To check if P1 can be transformed by the enzyme into P2, 25 μM P1 was incubated with deoxycholate and 1.5 units of LOX-1 in the standard reaction medium. Under these conditions an increase in absorbance at 234 nm is observed (data not shown). The products of this reaction were extracted and injected into a SP-HPLC column and eluted with *n*-hexane/2-propanol/H_2O. A single peak, with the same retention time of P2, was obtained (data not shown). The oxidation rate of P1 is comparable to that measured using DL-PC as a substrate (Table I).

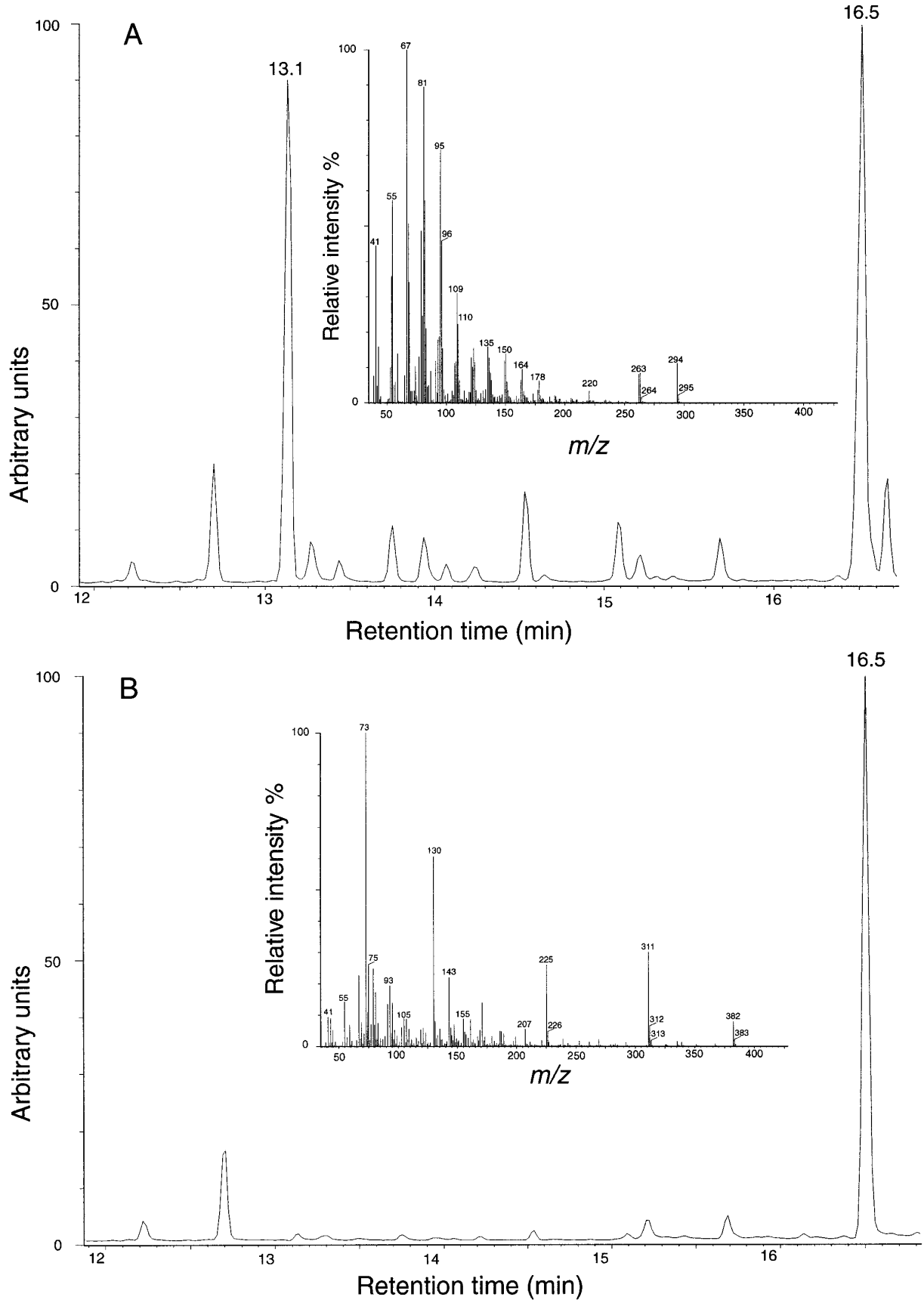
If P1 can be converted into P2 by LOX-1, it is likely that P1 corresponds to a molecule of DL-PC in which one of the linoleoyl chains is still available to the enzyme while the other has already been converted into HPOD. In P2, both linoleic acid units are probably converted into hydroperoxides. To check this hypothesis, P1 and P2 were reduced and transesterified and the resulting compound was trimethylsilylated and subjected to GC/MS. In the chromatogram displayed in Fig. 3A corresponding to the analysis of P1, two main peaks were detected: the mass spectrum of the peak at 13.1 min corresponds to the linoleoyl derivative (Fig. 3A, inset); the mass spectrum of the peak at 16.5 min (Fig. 3B, inset) showed the molecular ion at *m/z* 382 and fragment ions at *m/z* 311 and *m/z* 225. The ratio *m/z* 311 to *m/z* 225 is characteristic of the trimethylsilyl derivative of 13-HOD methyl ester (4). The GC/MS analysis of P2 (Fig. 3B) shows only the peak corresponding to the 13-HOD derivative. The same results were obtained when LOX-1 was incubated with DL-PC in phosphate buffer, pH 7.5 (data not shown).

The transesterification gives no information on the position (*sn1* or *sn2*) of the hydroperoxide. To check if the enzyme shows a preference for any of the linoleoyl chains, P1 was used as a substrate for PLA_2. The products of this reaction were extracted with a C18 column and injected on a SP-HPLC column. Figure 4 shows that both lysophosphatidyl choline and lysohydroperoxy phosphatidylcholine were obtained. These results imply that lipoxygenase oxidizes the linoleoyl chain at either the *sn1* or *sn2* position of glycerol.

DISCUSSION

When soybean LOX-1 is incubated with DL-PC in the presence of 10 mM sodium deoxycholate, an increase in absorbance at 234 nm is observed (Fig. 1B, trace 1). The repetitive scan for this reaction (Fig. 1A) is similar to that obtained when linoleic acid is used as a substrate. The lack of absorbance in the 270- to 280-nm region (Fig. 1A) indicates that, in contrast with the results reported by Eskola and Laakso (1983) (12), under our experimental conditions no oxodienes are formed. The progress curve at 234 nm (Fig. 1B) suggests that the reaction proceeds until the depletion of the substrate. The analysis of Fig. 2 indicates that the first product that appears is

FIG. 3. (A) Gas chromatogram of P1 and EI mass spectrum (inset) of the peak with a retention time of 13.1 min. (B) Gas chromatogram of P2. In the inset the EI mass spectrum of the peak with retention time of 16.5 min is shown. Prior to the analysis the samples were reduced, transesterified, and trimethylsilylated (see Materials and Methods for details).


A
13.1
16.5
Arbitrary units
Retention time (min)
Relative intensity %
m/z
B
16.5
Arbitrary units
Retention time (min)
Relative intensity %
m/z

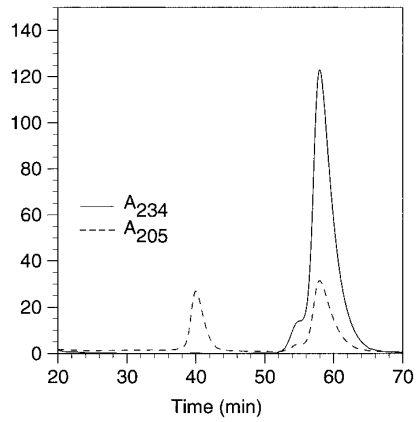


FIG. 4. Analysis of P1 with PLA_2. P1 was incubated for 5 min at RT and with constant stirring with PLA_2 in the presence of ether, Tris, and $CaCl_2$. The products were extracted with a C18 column and injected on SP-HPLC. Hydroperoxides and free fatty acids are eluted and lysophosphatidylcholine and lysohydroperoxy phosphatidylcholine are retained by the column.

the monohydroperoxy-linoleoyl phosphatidylcholine (P1), but its relative area never reaches 20%, because after 2.5 min the amount of P1 starts to decrease. The formation of the dihydroperoxy-linoleoyl phosphatidylcholine (P2) shows a different trend: after a short lag period its production increases linearly until a steady state is reached. After 25 min both P1 and PC are exhausted and only one final product (P2) is observed. The fact that P1 is rapidly converted into P2 and that the final product (P2) starts to appear already during the first minutes of the reaction when the amount of PC is still around 80% of the initial concentration suggests that both P1 and DL-PC are competing for the enzyme. The spectrophotometric measurement of the oxidation rate of DL-PC does not give information about which substrate is preferred by the enzyme as both the intermediate and the final products absorb at 234 nm. The dependence of the oxidation of phospholipids by soybean LOX on the presence and concentration of deoxycholate (12) could be the result of an enhancement in the susceptibility of the DL-PC toward the enzymatic attack rather than by an effect of the detergent on the enzyme. The results reported by Pérez-Gilabert *et al.* (16) and Schilstra *et al.* (17) strongly favor a model in which lipoxygenase has a substantially higher affinity for monomeric substrate than for free fatty acid incorporated into micelles. Thus, the lower activity displayed by soybean LOX-1 toward phospholipids could be the result of the micellar structure adopted by the substrate (18) which could affect the conformational flexibility of the protein. According to this model, at the beginning of the reaction both linoleoyl chains of DL-PC are available for the enzyme. The introduction of one hydroperoxide group in one of the chains may produce a disruption in the structure of the mixed bilayer disk, similar to the situation described by other authors when biological membranes are dioxygenated by lipoxygenase (4, 10). This new structure seems to make the remaining fatty acid more accessible to the action of LOX and P1 is thus transformed into P2. This is supported by the fact that the introduction of P1 into the reaction medium stimulates the oxidation rate of DL-PC. This effect cannot be attributed to the activation of the enzyme by HPOD as its inclusion in the reaction medium gives rise to a decrease in the initial velocity (Table I).

When acting on free linoleic acid, soybean LOX-1 produces two chiral compounds, 13*S*-HPOD and 9*S*-HPOD. The former of these hydroperoxides is generated over a broad pH range, while 9*S*-HPOD is not formed at pH levels above 8.5. According to Gardner (19), the nonionized carboxylic acid form of linoleic acid may arrange itself at the active site in two opposite orientations giving rise to either 9*S*-HPOD or 13*S*-HPOD. Thus, the exclusive production of the 13-HPOD derivative by soybean LOX-1 acting on DL-PC (Fig. 3) may be due to only one possible orientation mode as a result of the esterification. This is in-line with observations reported by Holtman *et al.* for barley LOX (20, 21). The incubation of linoleic acid with extracts from germinating barley embryos was found to give rise to the production of 9-HPOD as the main product; however, when acting on esterified lipids the main LOX product was 13-HPOD.

ACKNOWLEDGMENT

M.P.G. holds a postdoctoral contract from the European Commission (Fair CT965010).

REFERENCES

1. Yamamoto, S. (1992) *Biochim. Biophys. Acta* **1128,** 117–131.
2. Siedow, J. N. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42,** 145–188.
3. Dennis, E. A. (1987) *Drug. Dev. Res.* **10,** 205–220.
4. Kühn, H., Belkner, J., Wiesner, R., and Brash, A. R. (1990) *J. Biol. Chem.* **265,** 18351–18361.
5. Brash, A. R., Ingram, C. D., and Harris, T. M. (1987) *Biochemistry* **26,** 5465–5471.
6. Jung, G., Yang, D. C., and Nakao, A. (1985) *Biochem. Biophys. Res. Commun.* **130,** 559–566.
7. Takahashi, Y., Glasgow, W. C., Suzuki, H., Taketani, Y., Yamamoto, S., Anton, M., Kühn, H., and Brash, A. R. (1993) *Eur. J. Biochem.* **218,** 165–171.
8. Rapoport, S. M., and Schewe, T. (1986) *Biochim. Biophys. Acta* **864,** 471–495.
9. Conrad, D. J., Kühn, H., Mulkins, M., Highland, E., and Sigal, E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 217–221.
10. Feussner, I., Wasternack, C., Kindl, H., and Kühn, H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11849–11853.
11. Kondo, Y., Kawai, T., Hayashi, M., Ohnishi, T., Miyazawa, S., Itoh, T., and Mizutani, J. (1993) *Biochim. Biophys. Acta* **1170,** 301–306.

12. Eskola, J., and Laakso, S. (1983) *Biochim. Biophys. Acta* **751,** 305–311.
13. Finazzi-Agrò, A., Avigliano, L., Veldink, G. A., and Vliegenthart, J. F. G. (1973) *Biochim. Biophys. Acta* **326,** 462–470.
14. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917.
15. Van den Bosch, H., Aarsman, A. J., and Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta* **348,** 197–207.
16. Perez-Gilabert, M., Sanchez-Ferrer, A., and Garcia-Carmona, F. (1992) *Biochem. J.* **288,** 1011–1015.
17. Schilstra, M. J., Veldink, G. A., and Vliegenthart, J. F. G. (1994) *Lipids* **29,** 225–231.
18. Mazer, N. A., Benedek, G. B., and Carey, M. C. (1980) *Biochemistry* **19,** 601–615.
19. Gardner, H. W. (1989) *Biochim. Biophys. Acta* **1001,** 274–281.
20. Holtman, W. L. (1997) Ph.D. thesis, pp. 131–132, University of Leiden, Leiden, The Netherlands.
21. Holtman, W. L., Van Duijn, G., Sedee, N. J. A., and Douma, A. C. (1996) *Plant Physiology* **111,** 569–576.