Inhibition of Soybean Lipoxygenase-1 by Chain-Breaking Antioxidants

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ABSTRACT: The aim of this investigation was to determine whether chain-breaking antioxidants able to prevent lipid peroxidation can inhibit lipoxygenase-1 (EC 1.13.11.12). Therefore, the effects of ascorbic acid, 6-palmitoylascorbic acid and trolox on the enzyme activity were analyzed by means of Lineweaver–Burk double reciprocal plots and Yoshino's graphical method. The effect of these compounds on the formation of free radicals during lipoxygenase-1 reaction was investigated as well, by monitoring the enzymic formation of oxodienes. We present evidence that the chain-breaking antioxidants ascorbic acid, 6-palmitoylascorbic acid and trolox inhibit soybean lipoxygenase-1 in the micromolar concentration range (Ki 27, 3 and 18 μM, respectively). The inhibition is competitive, complete and reversible. All three compounds trap the free radicals formed during the lipoxygenase-catalyzed reaction, which might substantially contribute to their inhibitory ability. These findings can have physiological significance in the light of the lipoxygenase involvement in biomembrane remodelling.
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Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are nonheme, nonsulfur–iron dioxygenases which act on fatty acids containing one or more 1,4-*Z,Z*-pentadiene moieties to form *Z,E*-conjugated hydroperoxides. Lipoxygenases from both animal and plant tissues have been purified and characterized (*cf.* Refs. 1–3 for reviews). These enzymes play several physiological roles (1–3), in particular the product hydroperoxides are precursors of specific regulatory molecules, such as leukotrienes and lipoxins in animals (3,4), and jasmonic acid and traumatin in plants (2,5). Since the discovery of the role of lipoxygenases in the biosynthesis of potent effectors critical in animal and plant physiology, extensive research was done to find efficient inhibitors of their activity. Most studies have used soybean lipoxygenase-1 (LOX), a homologue of mammalian lipoxygenases whose three-dimensional structure (6,7) and catalytic mechanism (8,9) have been characterized in detail. LOX activity is suppressed by several compounds, which act in different ways on the LOX-catalyzed reaction (10, 1). This paper describes the ability of the antioxidants ascorbic acid (12,13), 6-palmitoylascorbic acid (13) and trolox (a water-soluble analogue of α-tocopherol) (12) to competitively inhibit LOX activity. Keeping in mind the role of LOX in biomembrane alteration (14–16), it is suggested that chain-breaking antioxidants can protect cell membranes from lipid peroxidation (12,13,17) through the inhibition of the LOX-catalyzed oxygenation of polyenoic fatty acids.

MATERIALS AND METHODS

Chemicals were of the purest analytical grade. Linoleic acid, ascorbic acid and sodium dithionite were from Sigma (St. Louis, MO). 6-o-Palmitoyl-L-ascorbic acid was purchased from Fluka (Buchs, Switzerland) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Janssen (Geel, Belgium).

LOX was purified from soybean [*Glycine max* (L.) Merrill, var. Williams] seeds according to Finazzi Agrò *et al.* (18). The purified enzyme showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had a specific activity of 200 μmoles O_2.min^{-1}.mg $protein^{-1}$, assayed polarographically (19). In the inhibition experiments, LOX activity was determined spectrophotometrically by recording the formation of conjugated hydroperoxides at 234 nm (19). A stock solution of linoleic acid (30 μM), dissolved in 1% Tween-20 in water, was diluted to the appropriate final concentration in 0.1 M sodium phosphate buffer, pH 6.8, according to Lomnitski *et al.* (20). Dehydroascorbic acid was biosynthesized by incubating ascorbic acid with an excess of ascorbate oxidase, as reported Maccarrone *et al.* (21). The incubation mixture was filtered through centricon 30 microconcentrators (Amicon, Lexington, MA), in order to remove the enzyme molecules.

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Abbreviation: LOX, lipoxygenase-1.

The effect of inhibitors on linoleic acid oxygenation by LOX was analyzed by Lineweaver–Burk double reciprocal plots (20). Enzyme inhibition was also analyzed by Yoshino's graphical method (22) in order to distinguish partial from complete inhibitors. We observed that the residual activity, when related to the activity in the absence of the inhibitor, was independent of the sequence of reagents addition. Therefore, the reaction was started by addition of the substrate (in the range 15–120 μM) to the reaction mixture containing 15 nM LOX and different inhibitor concentrations, namely 0–350 μM ascorbic acid, 0–35 μM 6-palmitoylascorbic acid, 0–50 μM trolox or 0–100 μM sodium dithionite. Reversibility of inhibition (11) was assessed by assaying residual LOX activity after dialysis of the enzyme/inhibitor mixtures (1 mL) against 0.1 M sodium phosphate buffer, pH 6.8 (100 mL), at 4°C. The pH dependence of LOX inhibition was determined as reported (20,23), using 90 μM linoleic acid and 15 nM LOX. Progress curves of the oxygenation of 30 μM linoleic acid catalyzed by 15 nM LOX were recorded by following the formation of the hydroperoxide products at 234 nm (19). Polarographic measurements of LOX activity (19) always confirmed the spectrophotometric data.

The effect of inhibitors on the formation of side products of linoleic acid oxygenation was assessed by following the formation of total oxodienes (i.e., 13-oxo-octadeca-9,11-dienoic plus 13-oxo-trideca-9,11-dienoic acids) at 285 nm (10,24). The standard reaction mixture contained 90 μM linoleic acid, 15 nM LOX and different amounts of inhibitors, in 0.1 M sodium phosphate buffer, pH 6.8. The data reported in this paper are the mean of three independent determinations (coefficient of variation <7%).

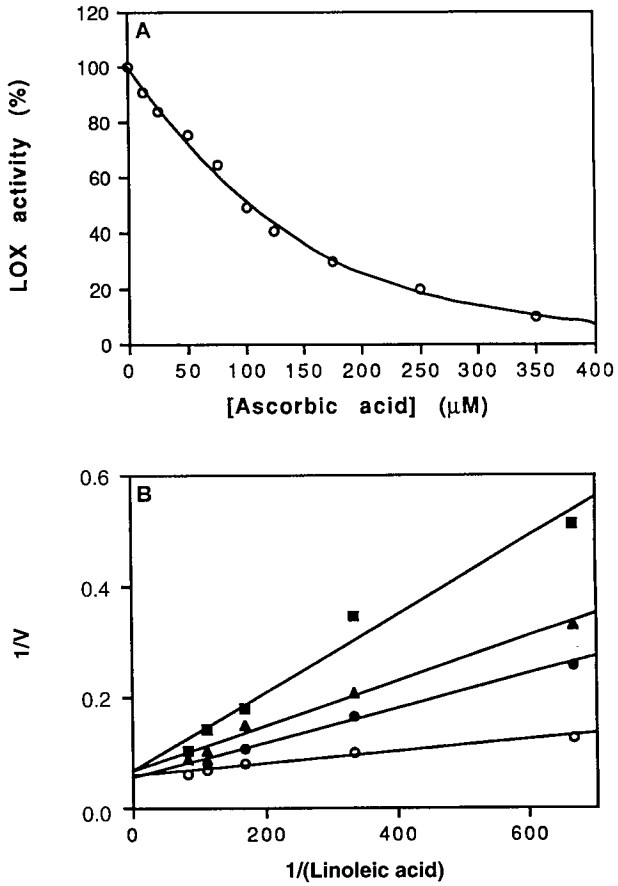

FIG. 1. A. Concentration-dependence of lipoxygenase-1 (LOX) inhibition by ascorbic acid. B. Lineweaver–Burk plots of linoleic acid oxygenation by LOX in the absence of inhibitors (open circles) or in the presence of 30 μM trolox (closed circles), 100 μM ascorbic acid (closed triangles) or 10 μM 6-palmitoylascorbic acid (closed squares). 1/V and 1/(Linoleic acid) values are expressed as μM^{-1}.min and $\mu M^{-1} \times 10^4$, respectively.

RESULTS

Ascorbic acid suppressed LOX-catalyzed oxygenation of linoleic acid in a concentration-dependent manner (Fig. 1A). Double reciprocal plots for LOX in the presence and absence of ascorbic acid are shown in Figure 1B.

The Michaelis constant was found to be 19 μM for linoleate in the absence of inhibitor, a value which agrees well with previous determinations (8). Ascorbic acid proved to be a competitive inhibitor of LOX (Fig. 1B), its inhibition constant, Ki, being 27 μM. In order to ascertain whether ascorbic acid inhibition was complete or partial, Yoshino's fractional-velocity plot was drawn (22). This plot (not shown) was consistent with a complete competitive inhibition. Moreover, ascorbic acid acted as a reversible inhibitor, with LOX regaining full activity after four minutes of dialysis of enzyme/inhibitor mixtures (15 nM/100 μM) at 4°C. The ability of ascorbic acid to inhibit linoleic acid oxygenation by LOX was dependent on the pH of the reaction, increasing from pH 5.5 to pH 7.0, and then dropping down between pH 7.0 and 9.0 (Fig. 2A). Finally, ascorbic acid inhibited the formation of oxodienes during the LOX-catalyzed reaction in a concentration-dependent way (Fig. 2B).

The inhibition of oxodiene formation was greater than the inhibition of linoleic acid oxygenation (compare Fig. 1A with Fig. 2B). At variance with ascorbic acid, dehydroascorbic acid was unable to inhibit oxodiene formation and inhibited LOX activity to a lesser extent. In fact, 150 μM dehydroascorbic acid reduced linoleic acid oxygenation to 70% of the control value, compared to 38% obtained with 150 μM ascorbic acid. The same inhibition studies performed with ascorbic acid were done with the chain-breaking antioxidants 6-palmitoylascorbic acid and trolox. Both compounds were found to be complete, reversible and competitive inhibitors of LOX activity (Fig. 1B), Ki values being 3 and 18 μM, respectively (Table 1). Sodium dithionite, a nonspecific reductant, slowed down LOX-catalyzed reaction by acting as a noncompetitive inhibitor, with a Ki of 49 μM (Table 1).

Interestingly, sodium dithionite inhibition resulted in a lengthening of the lag phase (9,19) of linoleic acid oxygenation (Fig. 3A), at variance with ascorbic acid (Fig. 3B) and the other chain-breaking antioxidants (data not shown).

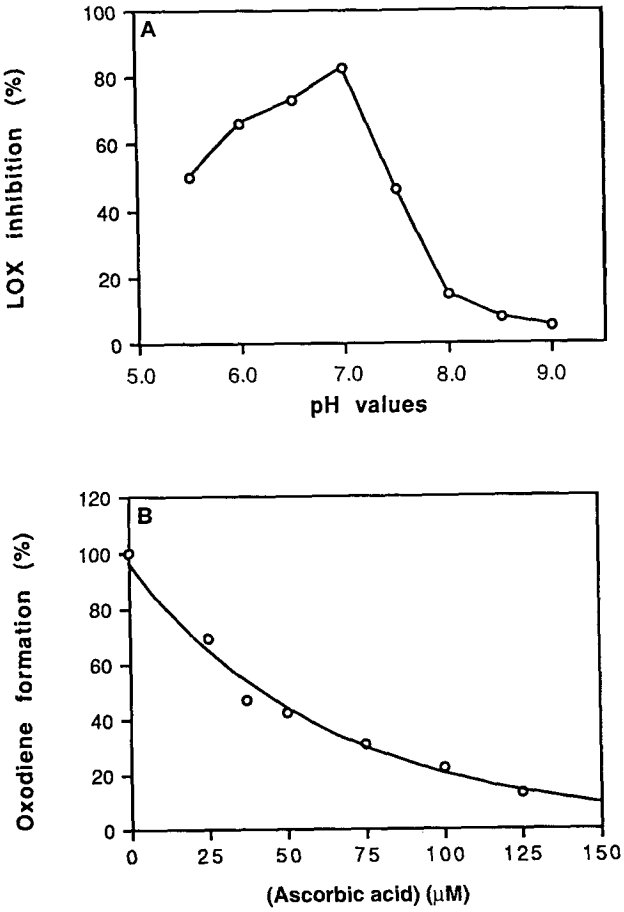

FIG. 2. A. Effect of pH on the inhibition of lipoxygenase-1 (LOX) activity by 250 μM ascorbic acid. B. Oxodiene formation in the presence of different amounts of ascorbic acid.

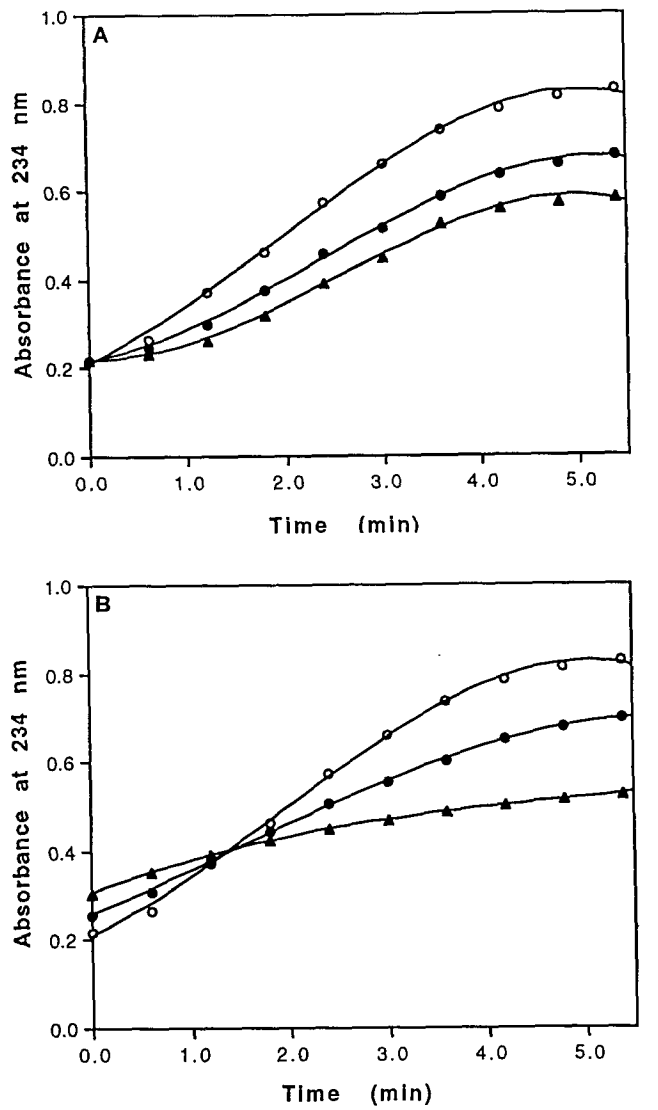

FIG. 3. A. Progress curves of LOX-catalyzed reaction, in the absence (open circles) or in the presence of 25 μM (closed circles) and 50 μM (closed triangles) sodium dithionite. B. Progress curves of LOX reaction in the absence (open circles) or in the presence of 50 μM (closed circles) and 100 μM (closed triangles) ascorbic acid. Abbreviation as in Figure 1.

TABLE 1
Inhibition of Lipoxygenase-1 by Chain-Breaking Antioxidants and Sodium Dithionite

Inhibitor	Inhibition type	Inhibition constant (Ki, μM)
Ascorbic acid	Competitive	27
6-Palmitoylascorbic acid	Competitive	3
Trolox	Competitive	18
Sodium dithionite	Noncompetitive	49

DISCUSSION

Oxygenation of linoleic acid catalyzed by soybean LOX is most effective at pH 9.0–10.0 (8,19). However, there was no inhibition of LOX under alkaline conditions (Fig. 2A). Conformational changes in the enzyme molecule might be responsible for the pH dependency of inhibition, a finding already reported for LOX (20,25). Therefore, experiments were performed at pH 6.8, where LOX retained high activity (26) and maximal inhibition was observed (Fig. 2A).

Several studies on soybean LOX showed that inhibitors can act through a number of mechanisms, e.g., by binding to sites other than the active site of the enzyme molecule (20), by preventing the formation of the activated Fe(III) form of LOX (27) or by reducing the catalytically active ferric enzyme to its inactive ferrous form through the formation of free radical metabolites (26). Here, ascorbic acid, 6-palmitoylascorbic acid and trolox are shown to act as competitive, complete and reversible inhibitors of LOX. Unlike other antioxidant inhibitors of LOX (26), the compounds under study did not reduce Fe(III) to inactive Fe(II), in fact the lag phase of the reaction (Fig. 3B) was not prolonged (9,10,19). Electron paramagnetic resonance spectroscopy (not shown) confirmed this finding, clearly indicating the lack of radical formation from the inhibitor molecules during LOX inhibition. At variance with the chain-breaking antioxidants, sodium dithionite acted as a noncompetitive inhibitor and caused a lengthening

of the lag phase of the reaction (Fig. 3A). This is consistent with sodium dithionite being a nonspecific reductant, which turns LOX into the inactive Fe(II) form (27). It can be suggested that the chain-breaking antioxidants might interact with the interior of the "funnel" leading to the active site (6), because enhancing the lipophilicity of the inhibitor made it more effective (Table 1). This hypothesis is in line with previous work on the competitive inhibition of LOX by *n*-alcohols, where longer alkyl chains were shown to increase the affinity of these compounds for the enzyme (28). Furthermore, ascorbic acid, 6-palmitoylascorbic acid and trolox are powerful radical-trapping antioxidants (12,13). Ascorbic acid strongly suppressed the formation of oxodienes (Fig. 2B), side products of linoleic acid oxidation which are indicative of the dissociation of free radicals from the active site (10,24). The same was found with 6-palmitoylascorbic acid and trolox (data not shown). These results suggest that the inhibitors can trap the radical intermediates escaping from LOX after hydrogen abstraction from linoleic acid, before enzymic oxygen insertion has occurred (8,10). Such radical-trapping ability might substantially contribute to the inhibition power of the compounds, as suggested also by the pH dependence (Fig. 2A). In fact, at neutral pH values, LOX produces more radicals than at pH 9.0, and trapping of these radicals, together with conformational changes of the enzyme (20), might improve LOX inhibition. The hypothesis that both hindrance of the active site and trapping of free radicals can contribute to LOX inhibition is also corroborated by the experiments with dehydroascorbic acid. The oxidized form of ascorbic acid was unable to reduce oxodiene formation (i.e., to trap radical intermediates), which resulted in a weaker inhibition of the LOX-catalyzed reaction. Finally, it seems noteworthy that trolox acted as competitive inhibitor of soybean LOX, at variance with its water-insoluble analogue α-tocopherol which inhibited the enzyme in a noncompetitive way (20). This difference resembles that of other compounds, e.g., retinol and β-carotene (20), and can be attributed to the different polarity of the two molecules.

In conclusion, evidence is presented that ascorbic acid, 6-palmitoylascorbic acid and trolox act as competitive inhibitors of soybean LOX. Thus, the effects of these chain-breaking antioxidants in protecting cell membranes by preventing lipid peroxidation can occur through the formation of an inactive complex with LOX, besides the trapping of free radicals. These results might have physiological significance in the light of lipoxygenase involvement in biomembrane alteration, both in animals (14) and in plants (15,16).

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REFERENCES

1. Vliegenthart, J.F.G., and Veldink, G.A. (1982) in *Free Radicals in Biology* (Pryor, W.A., ed.) pp. 29–64, Academic Press, New York.
2. Siedow, J.N. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42,* 145–188.
3. Yamamoto, S. (1992) *Biochim. Biophys. Acta 1128,* 117–131.
4. Samuelsson, B., Dahlem, S.E., Lindgren, S.E., Rouzer, C.A., and Serhan, C.N. (1987) *Science 237,* 171–176.
5. Hildebrand, D.F. (1989) *Physiol. Plant. 76,* 249–253.
6. Boyington, J.C., Gaffney, B.J., and Amzel, L.M. (1993) *Science 260,* 1482–1486.
7. Minor, W., Steczko, J., Bolin, J.T., Otwinowski, Z. and Axelrod, B. (1993) *Biochemistry 32,* 6320–6323.
8. Schilstra, M.J., Veldink, G.A., Verhagen, J., and Vliegenthart, J.F.G. (1992) *Biochemistry 31,* 7692–7699.
9. Schilstra, M.J., Veldink, G.A., and Vliegenthart, J.F.G. (1993) *Biochemistry 32,* 7686–7691.
10. Veldink, G.A., and Vliegenthart, J.F.G. (1991) in *Studies in Natural Products Chemistry* (Atta-ur-Rahman, ed.) Vol. 9, pp. 559–589, Elsevier, Amsterdam.
11. Cucurou, C., Battioni, J.P., Thang, D.C., Nam, N.H., and Mansuy, D. (1991) *Biochemistry 30,* 8964–8970.
12. Doba, T., Burton, G.W., and Ingold, K.U. (1985) *Biochim. Biophys. Acta 835,* 298–303.
13. Liu, Z.-L., Han, Z.-X., Chen, P., and Liu, Y.-C. (1990) *Chem. Phys. Lipids 56,* 73–80.
14. Kuhn, H., and Brash, A.R. (1990) *J. Biol. Chem. 265,* 1454–1458.
15. Kondo, Y., Kawai, Y., Hayashi, T., Ohnishi, M., Miyazawa, T., Itah, S., and Mizutani, J. (1993) *Biochim. Biophys. Acta 1170,* 301–306.
16. Maccarrone, M., Van Aarle, P.G.M., Veldink, G.A., and Vliegenthart, J.F.G. (1994) *Biochim. Biophys. Acta 1190,* 164–169.
17. Nagao, A., and Terao, J. (1990) *Biochem. Biophys. Res. Commun. 172,* 385–389.
18. Finazzi Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1973) *Biochim. Biophys. Acta 326,* 462–470.
19. Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1977) *Prog. Chem. Fats Other Lipids 15,* 131–166.
20. Lomnitski, L., Bar-Natan, R., Sklan, D., and Grossman, S. (1993) Biochim. *Biophys. Acta 1167,* 331–338.
21. Maccarrone, M., Rossi, A., D'Andrea, G., Amicosante, G., and Avigliano, L. (1990) *Anal. Biochem. 188,* 101–104.
22. Yoshino, M. (1987) *Biochem. J. 248,* 815–820.
23. Maccarrone, M., D'Andrea, G., Salucci, M.L., Avigliano, L., and Finazzi Agrò, A. (1993) *Phytochemistry 32,* 795–798.
24. Garssen, G.J., Vliegenthart, J.F.G., and Boldingh, J. (1971) *Biochem. J. 122,* 327–332.
25. Cucurou, C., Battioni, J.P., Daniel, R., and Mansuy, D. (1991) *Biochem. Biophys. Acta 1081,* 99–105.
26. Van der Zee, J., Eling, T.E., and Mason, R.P. (1989) *Biochemistry 28,* 8363–8367.
27. Sud'ina, G.F., Mirzoeva, O.K., Pushkareva, M.A., Korshunova, G.A., Sumbatyan, N.V., and Varfolomeev, S.D. (1993) *FEBS Lett. 329,* 21–24.
28. Kuninori, T., Nishiyama, J., Shirakawa, M., and Shimoyama, A. (1992) *Biochim. Biophys. Acta 1125,* 49–55.

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