Protection by Different Agents Against Inactivation of Lipoxygenase by Hydrogen Peroxide

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ABSTRACT: H_2O_2 is a potent inactivator of lipoxygenase. In this paper, the ability of different agents [mannitol, oleic, stearic and linoleic acid, *n*-butanol, and hydroperoxy octadecadienoic acid (HPOD)] to prevent the inactivation of tomato lipoxygenase by hydrogen peroxide has been studied. The involvement of $OH^{\cdot}$ in the inactivation process is suggested by the ability of mannitol to prevent the loss of activity. This radical would be produced by reaction of H_2O_2 with the Fe(II) lipoxygenase. The most effective protection was displayed by HPOD, the product of the reaction of lipoxygenase with linoleic acid. This result could be explained by the conversion of the native enzyme into the Fe(III) lipoxygenase in the presence of HPOD; the Fe(III) enzyme is not able to react with H_2O_2, and no $OH^{\cdot}$ will be produced. The protective effect obtained with oleic and stearic acid could be explained by an occupation of the active center by these inhibitors. The enzyme would not transform them, but their presence would hamper the conversion of H_2O_2 in $OH^{\cdot}$ and limit the damage in the active center.

Lipids 31, 1245–1250 (1996).

Lipoxygenases (LOX) (EC 1.13.11.12) catalyze the dioxygenation of fatty acids which contain one or more 1(*Z*), 4(*Z*)-pentadiene systems, yielding chiral (*E,Z*) conjugated hydroperoxy fatty acids (1). In mammalian tissues, LOX are involved in the initial steps of the biosynthesis of physiologically active compounds such as leukotrienes and lipoxins (2). The metabolic role of LOX in plants is still uncertain, although they have been implicated in plant senescence, in the response to pest attack as well as wounding (3,4) and in the formation of volatile flavor compounds in many plant food products (5,6).

The presence of one non-heme iron per enzyme molecule has been demonstrated in a number of LOX (7–9). From dormant soybean seeds, the enzyme is isolated as an EPR-silent Fe(II) form which is converted into the active Fe(III) form by the product hydroperoxide (10). The ability of other hydroperoxides such as H_2O_2 to convert the Fe(II)-LOX into the Fe(III) form has been studied by different groups. Some reports suggest that H_2O_2 does not activate the native enzyme (11,12), whereas other authors have demonstrated the ability of H_2O_2 to convert the soybean and reticulocyte 15-LOX from the Fe(II) into Fe(III) form (13,14). Low concentrations of H_2O_2 have been shown to stimulate the dioxygenase activity of soybean LOX (15) and 5-LOX from mammalian cells (16). H_2O_2 is also able to increase the hydroperoxidase activity of LOX, and it can replace lipid hydroperoxide in the oxidation of xenobiotics (17–19).

The irreversible inactivation of soybean LOX in the presence of H_2O_2 was reported in 1967 by Mitsuda *et al.* (20). The sensitivity of LOX to this agent is dependent on the degree of purification, the purified enzymes being more prone to inactivation by H_2O_2 than crude preparations (21). Kazeniac and Hall (22) and Jadhav *et al.* (23) reported the inhibition by H_2O_2 of the development of *cis*-3-hexenal and hexanal in blended tomatoes and in cell free tomato extracts. This data led them to suppose that LOX is involved in the sequence of reactions leading to hexanal and *cis*-3-hexenal formation from unsaturated fatty acids. Despite the fact that enzyme denaturation by H_2O_2 is not specific for LOX (17,24), the inhibitory effect of H_2O_2 has been taken as proof for the involvement of LOX in the flavor formation.

To gain further insight into the effect of hydroperoxides on LOX catalysis, we have studied the inactivation of tomato LOX by H_2O_2 and the influence of substrate, product, and inhibitors on the regulation of this process.

MATERIALS AND METHODS

Tomato LOX was purified using a modification of the method of Bonnet and Crouzet (25), (Suurmeijer, C.N.S.P., and Pérez-Gilabert, M., unpublished results). The specific activity of the enzyme was 10 enzymatic units per mg of protein. SDS-PAGE stained with Coomasie Blue showed three bands in addition to the corresponding to LOX.

Linoleic acid was purchased from Fluka (Bornem, Belgium); *n*-butanol was from Merck (Darmstadt, Germany); H_2O_2 30% wt was obtained from Aldrich (Bornem, Belgium); mannitol, stearic, and oleic acids were from Sigma (Bornem, Belgium). All other chemicals used were of analytical grade.

A H_2O_2 solution was freshly prepared every day, and its concentration was calculated using $\varepsilon_{240} = 39.4\ M^{-1}cm^{-1}$ (26).

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Abbreviations: HPOD, hydroperoxy octadecadienoic acid; LOX, lipoxygenase.

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Oleic, stearic, and linoleic acid were dissolved in methanol. The final concentration of methanol in the reaction medium was 1% (vol/vol).

9-hydroperoxy octadecadienoic acid (HPOD) was prepared by incubation of linoleic acid with tomato LOX in 0.1 M phosphate buffer pH 6.8 at 4°C and under constant aeration. The solution was applied to a 6 mL disposable C-18 extraction column (J.T. Baker Inc., Deventer, The Netherlands) and the product eluted with methanol. High-performance liquid chromatography (HPLC) (27) showed that the main product was 9-HPOD. 13-HPOD was prepared by incubation of linoleic acid with LOX-1 from soybeans in 0.1 M sodium borate buffer pH 10 and purified using the same procedure described for 9-HPOD.

LOX activity was determined spectrophotometrically at 234 nm [ε_{234} = 25,000 $M^{-1}cm^{-1}$, (28)] in a HP8452A diode array at 25°C. The rate was calculated from the linear zone of the product accumulation curve after the lag period.

The inactivation of the enzyme by hydrogen peroxide was confirmed determining the rate of oxygen consumption at 25°C. The equipment was the Hansatech CB1 Oxygen Electrode Unit equipped with magnetic stirrer and connected to a Kratos BD 40 recorder (Hansatech Limited, Norfolk, United Kingdom).

Unless stated otherwise, the reaction medium contained 0.1 M phosphate buffer pH 6.8, 18 μM linoleic acid and 0.016 enzyme units of tomato LOX in a final volume of 1 mL, these being the optimal conditions (Suurmeijer, C.N.S.P., and Pérez-Gilabert, M., unpublished results). One enzymatic unit is defined as the amount of enzyme that gives rise to the appearance of 1 μmol of conjugated diene per minute at 25°C.

In specific experiments, the Fe (III) LOX was obtained by preincubation of the native enzyme with an 80-fold excess of 9-HPOD for 20 min. The excess of hydroperoxide was eliminated by repeated centrifugations (at least four times) of the incubation medium through a Centriprep-30 concentrator (Amicon, IJsel, The Netherlands) as described in (29). The amount of HPOD introduced in the reaction medium along with the Fe(III) LOX was calculated to be less than $5 \cdot 10^{-5}$ μmoles.

RESULTS

The effect of H_2O_2 on the LOX activity was studied following the accumulation of HPOD in the presence of 1.5 mM H_2O_2 (Fig. 1A) and the oxygen consumption (Fig. 1B). The experiment was carried out in two different ways: (a) starting the reaction by addition of enzyme or by addition of substrate (b). Compared with a control without H_2O_2 (curve c), the presence of linoleic acid in the reaction medium prevents the loss of 22% of the activity. In order to exclude that this behavior is the result of a low sensitivity of tomato LOX against the action of H_2O_2, the enzyme was incubated with H_2O_2 at concentrations ranging from 50 to 150 μM (Fig. 2). Samples of the incubation media were taken at different times, and the residual activity was measured by the addition of linoleic acid to the cuvette. In Figure 2, it is shown that when the substrate is not present in the incubation medium, the inactivation of LOX by H_2O_2 is dependent on the concentration of H_2O_2 and on the duration of the incubation. This inactivation is similar

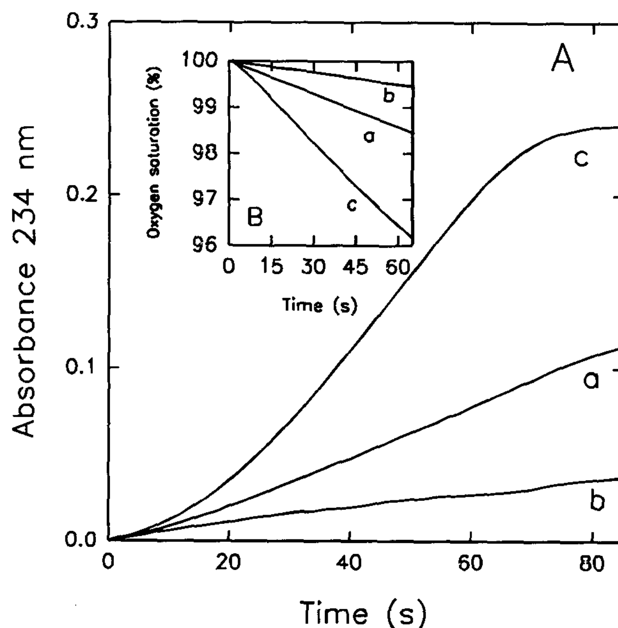

FIG. 1. (A) Progress curves for the oxidation of linoleic acid by tomato lipoxygenase in the standard reaction medium plus 1.5 mM H_2O_2 (traces a and b). The reaction started by the addition of the enzyme (a), or by the addition of the substrate (b). A control without H_2O_2 is shown in trace c. (B) The same experiment was repeated following the rate of O_2 consumption.

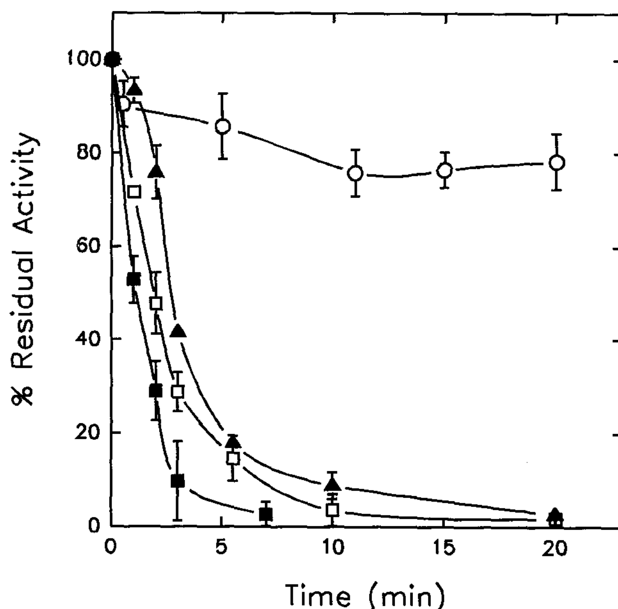

FIG. 2. Inactivation of tomato lipoxygenase by incubation with different concentrations of H_2O_2. The enzyme was incubated in the standard reaction medium containing: no H_2O_2 (○); H_2O_2 50 μM (▲); 75 μM (□), and 150 μM (■). The reaction started by the addition of linoleic acid. The mean value of three determinations ± σ_n is represented.

to that displayed by soybean LOX (20) and indicates that the sensitivity of both enzymes toward H_2O_2 is similar.

The results presented in Figures 1 and 2 suggest that the presence of linoleic acid somehow prevents the inactivation of tomato LOX by a high concentration of H_2O_2. This observation could be explained either by a direct effect of the substrate occupying the active center and thus preventing the attack of H_2O_2 on the catalytic site or by an effect of the product of the reaction.

To check if there was any relationship between the linoleic acid concentration and the percentage of the residual activity, the initial rate was measured in the presence of different concentrations of H_2O_2. The results were similar when different linoleic acid concentrations, i.e., 2.5 μM, 18 μM, or 36 μM were used (Fig. 3).

In order to find out if the product of the reaction played a role in the mechanism of protection, the enzyme was incubated with 2.5 μM 9-HPOD or 2.5 μM 13-HPOD, plus 50 μM H_2O_2 and the residual activity at different intervals of time was measured. From Figure 4, it can be concluded that the presence of either 13-HPOD (◆) or 9-HPOD (△) indeed protects the enzyme against denaturation.

This effect of HPOD can be explained by a physical protection of the active center or by a difference in the sensitivity of Fe(II)-LOX and Fe(III)-LOX to the action of H_2O_2. To distinguish between these two possibilities, the native Fe(II) LOX was preincubated for 20 min with 9-HPOD, and the excess of HPOD was removed by ultrafiltration (see the Materials and Methods section). The obtained Fe(III) enzyme was

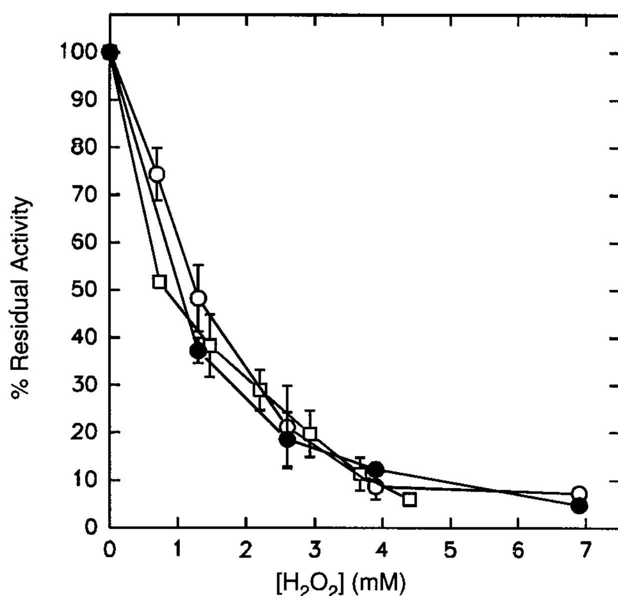

FIG. 3. Effect of the substrate on the inactivation of lipoxygenase by H_2O_2. The reaction medium contains different concentrations of H_2O_2, 0.016 enzymatic units of tomato lipoxygenase and 2.5 μM (□), 18 μM (○), or 36 μM (●) linoleic acid. The reaction started by the addition of lipoxygenase. The mean value of three determinations ± σ_n is represented.

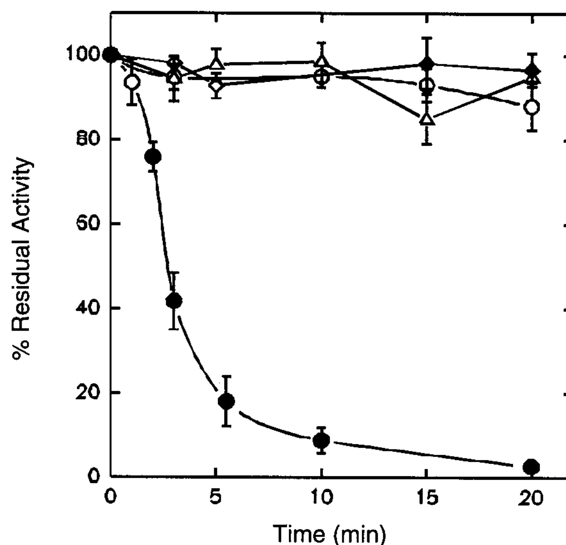

FIG. 4. Effect of HPOD on the inactivation of lipoxygenase by H_2O_2. Fe(II) (●) or Fe(III)-LOX (○) were incubated with 50 μM H_2O_2. Fe(III)-LOX was prepared as described in the Materials and Methods section. In another experiment, lipoxygenase was incubated in the presence of 50 μM H_2O_2 + 2.5 μM 13-HPOD (◆), or 2.5 μM 9-HPOD (△). In the three experiments, the reaction started by the addition of 18 μM linoleic acid. The mean value of three determinations ± σ_n is represented.

incubated with 50 μM H_2O_2, and the residual activity was measured. The results presented in Figure 4 indicate that there is no difference in activity between the experiment carried out with the enzyme preincubated with HPOD (○) or with the enzyme incubated with H_2O_2 + HPOD (◆ and △).

Early reports (20,30) described the differences in the protective effect displayed by certain competitive inhibitors against the inactivation of soybean LOX by H_2O_2. They observed that analogs of linoleic acid were able to protect the enzyme, whereas saturated monohydric alcohols were not effective. These differences were explained supposing that the linoleic acid analogs were able to occupy the active center, whereas the alcohols could merely attach to a hydrophobic region of the protein. In the present study, tomato LOX was incubated with H_2O_2 in the presence of different concentrations of stearic, oleic acid, or *n*-butanol (Fig. 5). It was found that oleic and stearic acids were able to prevent the inactivation when concentrations in the range of 36 μM were tested (no higher concentrations were used to avoid the turbidity of the reaction medium). However, *n*-butanol, even at a concentration of 0.2 M in the incubation medium, was ineffective in preventing the enzyme denaturation by H_2O_2 (Fig. 5). In the presence of 0.2M *n*-butanol, the residual activity is 58% with respect to a control without *n*-butanol.

It has been suggested (31) that the inactivation produced by H_2O_2 may be a consequence of reactive oxygen species such as $OH^{\cdot}$ produced by reaction with the Fe(II) in the enzyme. This radical may modify different amino acids in the active center of the enzyme such as histidine (20). The effect of mannitol, a well-known radical scavenger, (32) was also

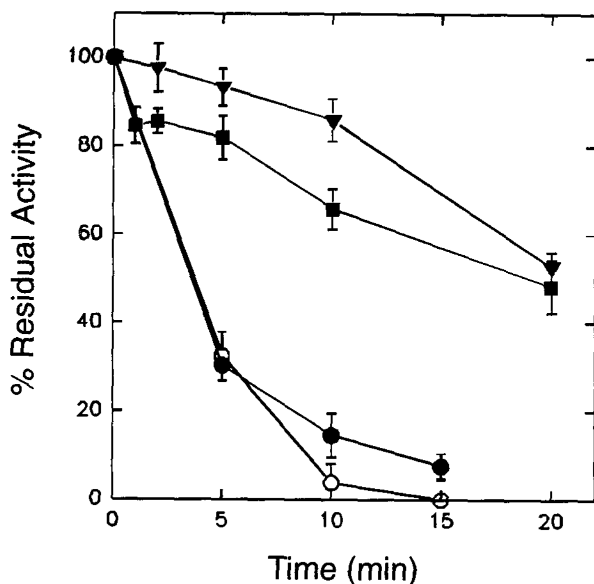

FIG. 5. Effect of different lipoxygenase inhibitors. The enzyme was incubated with 50 μM H_2O_2 plus 36 μM oleic acid (▼); 36 μM stearic acid (■); *n*-butanol 0.1M (○) or 0.2 M (●). The reaction started by the addition of 18 μM linoleic acid. The mean value of three determinations ± σ_n is represented.

tested, and it was found that only concentrations in the range of 0.2 M were able to partially prevent the inactivation (Fig. 6).

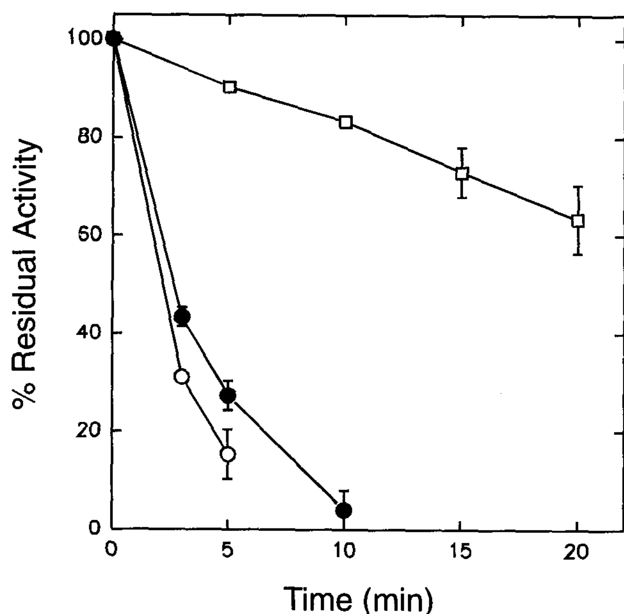

FIG. 6. Effect of mannitol. Lipoxygenase was incubated in the standard reaction medium with 50 μM H_2O_2 plus different concentrations of mannitol: 0.2 mM (○); 4 mM (●), and 0.2 M (□). The reaction started by the addition of 18 μM linoleic acid. The mean value of three determinations ± σ_n is represented.

DISCUSSION

The protective effect of different substrates on the hydroperoxidase activity of LOX against inactivation by H_2O_2 has been described recently (17–19). In the absence of a hydrogen donor, H_2O_2 causes a decrease in the rate of xenobiotic oxidation. In this paper we describe for the first time the protection of the dioxygenase activity of LOX by the substrate of the reaction.

The presence of linoleic acid in the reaction medium strongly prevents the inactivation of lipoxygenase by high concentrations of H_2O_2. In Figure 1A the progress curves for the oxidation of linoleic acid by LOX in the presence of 1.5 mM H_2O_2 are shown, thus indicating that the initial velocity depends on the order of addition of the reagents, namely first the substrate (trace a) or first the enzyme (trace b). When the reaction is started by the addition of substrate, the enzyme loses 87% of its initial activity in the time lapse between the addition of the enzyme and the addition of linoleic acid (about 10 s). However, when the reaction starts by the addition of LOX, the inactivation is 65%. The same results were obtained when the reaction was determined polarographically as the rate of O_2 consumption (Fig. 1B), thus confirming the inactivation of the enzyme.

When tomato LOX is preincubated with different concentrations of H_2O_2, a time-dependent inactivation is observed (Fig. 2). A concentration of 150 μM H_2O_2 abolishes the enzymatic activity in less than 8 min. This result indicates that the sensitivity of tomato LOX to hydrogen peroxide is similar to that reported by Mitsuda *et al.* (20) and Egmond *et al.* (31) for soybean lipoxygenase.

If the decrease in the inactivation shown in Figure 1 is a direct result of the presence of linoleic acid in the reaction medium, different responses should be expected depending on the concentration of the substrate. However, the results presented in Figure 3 show that the residual activity measured in the presence of different amounts of H_2O_2 is the same regardless of the linoleic acid concentration used (2.5, 18, or 36 μM). An alternative explanation is that the product of the reaction was the "real" responsible for the protection displayed by linoleic acid. To check this possibility, LOX was preincubated with 50 μM H_2O_2 and 2.5 μM 9-HPOD, the product of the tomato LOX reaction, or with 13-HPOD. The results presented in Figure 4 indicate that both 9- and 13-HPOD are able to avoid partially the inactivation of the enzyme. Egmond *et al.* (31) and Aoshima (33) reported a correlation between the fluorescence quenching and the inactivation of soybean LOX by H_2O_2. This quenching would be due to the conversion of Fe(II)-LOX into the Fe(III)-LOX (31):

$$\text{LOX-Fe(II)} + H_2O_2 \rightarrow \text{LOX-Fe(III)} + OH^{\cdot} + OH^- \quad [1]$$

The active oxygen species produced in this process would react with amino acids near or at the active site (34) inactivating the enzyme. Thus, the protection of LOX by HPOD (Fig. 4) could be explained by the conversion of the native enzyme in the Fe(III) LOX which, according to the above

scheme, is not able to react with H_2O_2. To test this hypothesis and to rule out a protection due to the attachment of the HPOD to the active center, the residual activity of Fe(III) enzyme after its incubation with 50 μM H_2O_2 was measured. In Figure 4 it is shown that while the native enzyme completely loses its activity after 20 min of incubation with H_2O_2 the Fe(III)-LOX retains its activity. This larger sensitivity of the Fe(II) toward H_2O_2 has been observed by Percival *et al.* (21) with purified human 5-LOX.

The model proposed in reaction 1 cannot explain the results obtained in this work with competitive inhibitors such as oleic and stearic acid (Fig. 5). In a recent paper, Van der Heijdt *et al.* (35) suggested the possibility that oleic acid would bind to the same site on LOX as linoleate. In this sense the protective effect obtained with oleic and stearic acid could be explained by an occupation of the active center by these inhibitors. The enzyme would not transform them, but their presence would hamper the conversion of H_2O_2 in $OH^{\cdot}$ and limit the damage in the active center. The effect of *n*-butanol, another competitive inhibitor of LOX, was also studied, but concentrations up to 0.2 M of this compound were unable to prevent the inactivation of tomato LOX by H_2O_2 (Fig. 5). This result is in accordance with the data reported for the soybean enzyme (30,36) and have been interpreted supposing that these alcohols attach to a site other than the catalytic site (30). Boyington *et al.* (37) described the existence of three hydrophobic cavities in LOX-1 from soybean; two of them connected to the iron site, while the largest cavity is not in contact with the active center. Though the three-dimensional structure of tomato LOX has not been established up until now, our results suggest the existence of similarities between tomato and soybean LOX.

The involvement of $OH^{\cdot}$ in the inactivation process suggested in reaction 1 is supported by the ability of mannitol to protect the enzyme from inactivation. In Figure 6, the results obtained by adding different amounts of mannitol to the incubation medium are shown. The high concentration of mannitol (0.2 M) needed to protect the enzyme against inactivation is explained considering that, in order to scavenge this $OH^{\cdot}$, the mannitol molecules must be present in the active center at the same time as H_2O_2, but the hydrophobicity of the active center makes the access of mannitol difficult. *In vivo*, an increase in the production of H_2O_2 has been observed after the addition of elicitors to plant cell cultures (38). This increase precedes LOX elicitation (39), suggesting that this enzyme may contribute to diminish the concentration of H_2O_2 at this metabolic stage.

The results in this paper suggest that the protective effect displayed by linoleic acid against the inactivation of tomato LOX by H_2O_2 may be the result of two different processes, one physical and the other chemical. The physical protection would be a consequence of the occupation of the active center by the substrate which would hinder the access of H_2O_2 to the catalytic site. This effect can be mimicked by those competitive inhibitors of LOX that attach to the active center. The chemical protection is produced by the HPOD synthesized by LOX or present in linoleic acid, and consists of the conversion of Fe(II)-LOX into the Fe(III) form which cannot catalyze reaction 1 and thus is insensitive to H_2O_2. Radical scavengers diminish LOX inactivation only if a high concentration is used. The lower sensitivity of crude extracts of LOX to H_2O_2 compared with purified enzyme preparations (21) could be explained by the presence in the extraction medium of "protective agents" such as substrates, competitive inhibitors, or radical scavengers that would be subsequently removed during the purification. The better understanding of the mechanism of LOX inactivation by H_2O_2, also may facilitate optimization of purification procedures.

ACKNOWLEDGMENTS

This work was partially supported by MEC (Spain) and European Commission (N° FAIR-CT96-5010).

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[Received June 10, 1996, and in final revised form October 7, 1996; Revision accepted October 14, 1996]