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Kinetic Properties of Lipoxygenase from Desert Truffle (*Terfezia claveryi* Chatin) Ascocarps: Effect of Inhibitors and Activators

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There is very little information available on the kinetic characteristics of fungal lipoxygenases (LOXs) because most data on the mechanism of this enzyme concern soybean LOX. In this paper, the kinetic properties of LOX from *Terfezia claveryi* Chatin ascocarps were studied for the first time. The enzyme did not show the "substrate aggregation-dependent activity" described for other LOXs and presented a K_m for linoleic acid of 41 μM at pH 7.0. The effect of different inhibitors was also studied. The enzyme presented the characteristic lag phase of other LOXs, and the influence of different factors on its duration was analyzed. The lag period was reduced not only by the product of the reaction (13-HPOD) but also by 9-HPOD. Calculation of the activation constant is proposed for the first time as a useful tool for the characterization of LOX because this method makes it possible to quantify the effectiveness of different hydroperoxides as LOX activators. The activation constants obtained were 0.3 and 6.4 μM for 13- and 9-HPOD, respectively; thus, the product of the reaction was ~21-fold more effective than 9-HPOD as a *T. claveryi* LOX activator.

KEYWORDS: Activation constant; ascocarp; desert truffle; hydroperoxide; kinetic mechanism; fungus; inhibitor; lag period; lipoxygenase; *Terfezia*; Triton X-114.

INTRODUCTION

Terfezia claveryi Chatin is an hypogeous ascomycete, which establishes mycorrhizal symbiosis with several annual and perennial species of the *Helianthemum* genus (*1*). Its edible ascocarps are rich in fiber, proteins, vitamins, and minerals (*2*, *3*), with high commercial value. There is a growing interest in introducing *T. claveryi* cultivation into dry environments as a useful way of exploiting lands that, until now, have been regarded as unproductive (*4*, *5*).

Oxidative and reductive processes play a key role in foods and influence not only the taste but also the color, texture, and nutritional value of food products. Two oxidases, tyrosinase and lipoxygenase (LOX), which may play an important role in some of these processes have been purified from *T. claveryi* ascocarps (*6*−*9*). LOXs are widely distributed in animals and plants, and their products have a wide range of biological functions (*10*). LOXs are involved in the development of oxidative rancidity in some foods (*11*) and may also contribute to the flavor formation in others (*12*, *13*). Despite the important role that this enzyme seems to play in the quality of edible fungi, there is little information on fungal LOX. The high lipid content of *T. claveryi* ascocarps (*3*) makes lipid rancidity, a process that may be accelerated by the presence of LOX, the main factor limiting their storage life.

LOXs (linoleate: oxygen oxidoreductase, EC 1.13.11.12) 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. Relatively little research has been done on the kinetics of LOX-catalyzed reactions, probably reflecting the complicated nature of the system. These complications include the low solubilities of both the fatty acid and O_2 substrates in aqueous systems, the purity of the fatty acid, enzyme inhibition by the fatty acid substrate, the effects of pH and temperature on positional and chiral specificities, and the inactivation of the enzyme during the reaction. LOX, both from plant and animal sources, exhibits a characteristic "induction period" (*14*) or "kinetic lag phase" (*15*), and after initiation of the reaction, the rate gradually increases until a maximum is reached. The origin of this rate increase has been the subject of much discussion. According to the two-step model proposed by the group of Veldink and Vliegenthart (*16*−*19*), only Fe^{3+} LOX can catalyze the first step of the reaction (**Scheme 1**). Because LOX in its resting state contains high-spin Fe^{2+} (*20*, *21*), the reaction is either started by traces of hydroperoxides (step A of **Scheme 1**), formed during autoxidation of the substrate, and/or by traces of the Fe^{3+} LOX present in the Fe^{2+}

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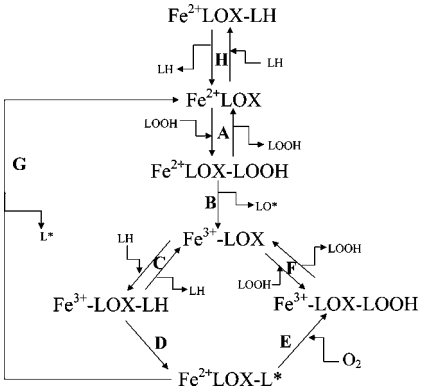
Scheme 1. LOX Reaction Mechanism (Adapted from Schilstra et al., 1992)[a]



[a] Abbreviations: LOOH, hydroperoxy fatty acid product; LH, fatty acid substrate; L* and LO*, oxidized and reduced forms of substrate and product (radical compounds).

LOX preparation (*22*). The two-step mechanism thus attaches a central role to the cycling between the Fe^{2+} and Fe^{3+} enzyme forms and predicts the occurrence of an induction period (*17*−*19*). Most data concerning the mechanism of the LOX reaction originates from soybean LOX-1, because it is abundant in the seeds, easy to purify, and its pH optimum (pH 9.0) facilitates preparation of aqueous solutions of substrates; there are very few studies on the kinetic properties of LOX from edible fungi.

LOX from *T. claveryi* ascocarps has been purified to apparent homogeneity by phase partitioning in the presence of TX-114, followed by two steps of cation-exchange chromatography (*9*). The enzyme consisted of a single major band with an apparent molecular mass of 66 kDa after SDS−PAGE. When the enzyme reacted with linoleic or linolenic acid, it produced a single peak that was identified as the 13-hydroperoxide of the corresponding fatty acid (*9*).

The aim of the research described in the present paper was to study the kinetic properties of LOX from *T. claveryi* ascocarps. The influence of different factors on the duration of the lag period and the effect of various compounds on LOX activity are also reported.

MATERIALS AND METHODS

Fungal Material. Ascocarps of *T. claveryi* were collected in Zarzadilla de Totana (Lorca, Murcia, Spain), associated with *Helianthemum almeriense* Pau shrubs, and used a few hours after collection or after storage at −20 °C.

Reagents. β-Cyclodextrin (β-CD) and esculetin were from Aldrich (Madrid, Spain). Bicinchoninic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), soybean LOX, and sodium ascorbate were purchased from Sigma (Madrid, Spain). Triton X-114 (TX-114), linoleic (LA), and NDGA were obtained from Fluka (Madrid, Spain). Potato LOX was purified following the method of Mulliez and coworkers (*33*). The remaining reagents were of analytical grade.

Enzyme Purification. The enzyme was purified as described elsewhere (*9*). In brief, pieces of *T. claveryi* ascocarps were homogenized at 4 °C after suspension in 0.1 M sodium phosphate buffer at pH 7.0 in a ratio of 1:5 (w/v). The homogenate was then centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant was subjected to temperature-phase partitioning by adding TX-114 (final concentration of 8%, w/v). The mixture was kept at 4 °C and then warmed to 37 °C until the solution became spontaneously turbid. This solution was centrifuged at 15000*g*, and the clear detergent-poor supernatant was subjected to a second temperature-phase partitioning by adding TX-114 (final concentration of 6%, w/v). After centrifugation (15000*g*, 15 min, 30 °C), aliquots of the supernatant were loaded onto a 1 mL Resource S column and equilibrated with 50 mM sodium phosphate buffer at pH 5.5. LOX activity was eluted from the column with a NaCl gradient from 0 to 1 M NaCl. Samples containing LOX were stabilized by adding TX-100 (final concentration of 0.04%, v/v).

Enzyme Activity Assays. *Determination of pH Optimum in the Presence of CDs.* To determine the effect of pH on LOX activity, the enzymatic activity was followed at 25 °C and the increase in absorbance at 234 was monitored in a Kontron spectrophotometer. To maintain the monomeric concentration of linoleic acid (LA_f) constant (7 μM) at each pH, different amounts of fatty acid were dissolved with 1 mM β-CD in the corresponding buffer. The total concentration of the linoleic acid (LA_t) necessary to yield the desired amount of LA_f at each pH was calculated using a set of constants determined in a previous work (*23*) and the following equation:

$$[LA]_t = [LA]_f(1 + K_1[CD] + K_1K_2[CD]^2) \quad (1)$$

The buffers used were 0.1 M sodium phosphate containing 1% ethanol for pH 5.0−7.6 and sodium borate containing 1% ethanol for pH 7.6−10.0. The complexes LA−β-CDs were prepared by dissolving β-CDs in the corresponding buffer containing 1% ethanol, followed by the addition of fatty acid prepared in the same buffer. The samples were flushed with N_2 to prevent LA oxidation during the preparation. The reaction was started by adding the enzyme to a cuvette containing the reaction medium. The reaction progress was followed spectrophotometrically at 234 nm ($\epsilon = 25\,000\ M^{-1}\ cm^{-1}$).

Effect of the Substrate Concentration. The effect of the LA concentration on LOX activity was determined by measuring oxygen consumption with a Clark-type electrode. The 1-mL samples consisted of 0.1 M phosphate buffer at pH 7.0, 1% (v/v) ethanol, and different concentrations of fatty acid. They were vigorously shaken before use so that they would become air-saturated and were transferred to the stirred, thermostated oxygraph chamber (Hansatech Ltd. Norfolk, U.K.) where the reaction was started by adding the enzyme.

K_m and V_{max} were calculated by nonlinear regression fitting (*24*) of the experimental points to the following equation:

$$V = \frac{V_{max}[\text{substrate}]}{K_m + [\text{substrate}]} \quad (2)$$

Inhibition Studies. The effect of different chemicals on the LOX activity was determined spectrophotometrically. The assays were conducted using 0.1 M borate buffer at pH 10.0 to avoid interferences because of the turbidity of the fatty acids. The compounds used were BHA, BHT, NDGA, esculetin, and sodium ascorbate. The antioxidants were solubilized in ethanol, except sodium ascorbate, which was prepared in 0.1 M sodium phosphate buffer at pH 7.0. The reaction medium contained 0.5 mM inhibitor and 180 μM LA; the reaction was started by adding the enzyme. The control assays were carried out using ethanol without an inhibitor.

The effect of NDGA incubation on the LOX activity was also studied. LOX was incubated with 0.1 mM NDGA in 0.1 M sodium borate buffer at pH 10.0. Aliquots were withdrawn at different intervals, and the reaction was started by adding LA (final concentration of 180 μM).

Effect of Different Factors on the Lag Phase. The reaction was followed spectrophotometrically measuring the increase in absorbance at 234 nm. To avoid interferences because of the low solubility of LA, the assays were carried out using 0.1 M sodium borate buffer at pH 10. When the effect of HPOD was studied, the reaction medium consisted of 10 μL of 18 mM LA in ethanol, 10 μL of HPOD, and 20 μL of LOX, in a final volume of 1 mL containing 0.1 M sodium borate buffer at pH 10. 13-HPOD was obtained by incubation of LA with soybean LOX in sodium borate buffer at pH 10, and 9-HPOD by incubating LA with 5-LOX from potato in 0.1 M phosphate buffer at pH 6.3; the reaction products were extracted with an octadecyl solid-phase column and analyzed by HPLC before use (the purity of each HPOD was >95%). The duration of the induction period was quantified by measuring the time axis intercept of a straight line through the portion of the reaction progress curve where the rate is maximal. The steady-state rate was calculated from the linear zone of the reaction progress curve after the induction period.

The experiments were performed in triplicate, and the mean and standard deviation were plotted.

RESULTS AND DISCUSSION

Effects of pH. It is well-known that pH determines not only the protonation but also the aggregation state of fatty acids (*25*). At pH 7.0, the LA cmc is approximately 20 μM (*26*). Thus, when LA concentrations below this value are used, the substrate is quickly consumed and it is difficult to measure the LOX activity in this region. On the other hand, when the LA concentration is above cmc, fatty acids form a micellar phase and scattering makes the spectroscopic determination of LOX activity similarly difficult.

To avoid these problems, the pH optimum was determined using a method based on the formation of the inclusion complex between PUFAs and CDs as previously reported by our group (*27*, *28*). The transparency of the inclusion-complex solution and the low absorption of β-CD, even at very low wavelengths, permitted UV characterization of the reaction. In addition, the use of CDs permits an increase in the total substrate concentration, while the free fatty acid concentration is kept constant (*27*, *28*). This increases the length of the linear region of the product accumulation curve without affecting the constant rate and consequently improves the determination of the LOX activity. We set the conditions (total LA and CDs) considered necessary to obtain a very low concentration of free substrate (7 μM). At this concentration, LA was in the monomeric form at every pH assayed. The pH profile depicted presents an optimum at pH 7.0 (data not shown), and it is typical of other LOXs such as ungerminated barley (*29*), eggplant fruit (*26*), and LOX from *Gäumannomyces graminis* (*30*).

Effect of Substrate Concentration. In spectrophotometric studies of the LOX-catalyzed reaction at neutral pH, the low solubility of fatty acids is a common problem. One way of overcoming visual turbidity is to use detergents (e.g., Tween-20) that form micelles, which solubilize and optically clear these fatty acid suspensions (*31*). Detergents can be added to the substrate (*32*) or to the buffer (*33*). Although both methods effectively prevent turbidity, the results obtained when the variation of the catalysis rate with substrate concentration was studied was found to be dependent on the reaction medium (*34*). In the present paper, the effect of linoleic acid concentration on LOX activity was carried out in 0.1 M sodium phosphate buffer at pH 7.0 in the absence of the detergent. To avoid interferences because of the substrate turbidity, the activity was determined measuring the oxygen consumption. The initial rate showed a hyperbolic dependence with respect to the substrate concentration (data not shown). The values obtained for V_{max} and K_m gave values of 19 μM/min of protein and 41 μM, respectively. This K_m is ~3-fold lower than that reported for LOX from *Pleurotus* at its pH optimum (pH 8.0) (*35*), but because the reaction medium contained Tween-20, the results cannot be properly compared. *T. claveryi* LOX does not show the "substrate aggregation-dependent activity" described for eggplant LOX (*26*) because the formation of LA aggregates does not affect the activity.

Effect of Inhibitors. The effect of different compounds (final concentration of 0.5 mM) on the LOX activity was studied. The results presented in the inset of **Figure 1** indicate that nordihydroguaiaretic acid (NDGA), a catecholic antioxidant, is an effective inhibitor of *T. claveryi*, decreasing its activity by 94% at 0.5 mM. Kemal and co-workers (*36*) demonstrated that this compound inhibited soybean LOX by reducing the catalytically active Fe^{3+}-LOX to the catalytically inactive Fe^{2+}-LOX. In addition to this reversible inhibition, the incubation of *T. claveryi* LOX with NDGA for long periods of time leads to irreversible inactivation of the enzyme (**Figure 1**). Different authors (*36*, *37*) suggest that this inactivation is caused by hydrogen peroxide, a known potent irreversible inactivator of LOX (*38*), formed by air oxidation of NDGA.

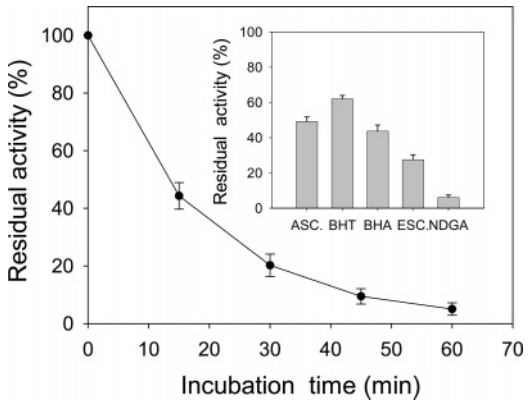


Figure 1. Effect of NDGA incubation on *T. claveryi* LOX activity. LOX was incubated with 0.1 mM NDGA in 0.1 M sodium borate buffer at pH 10.0. Aliquots of the incubation medium were withdrawn at different times, and the reaction was started by adding LA (180 μM in cuvette). Inset, effect of different compounds on LOX activity (see the Material and Methods for details). Abbreviations: ASC, sodium ascorbate; ESC, esculetin.

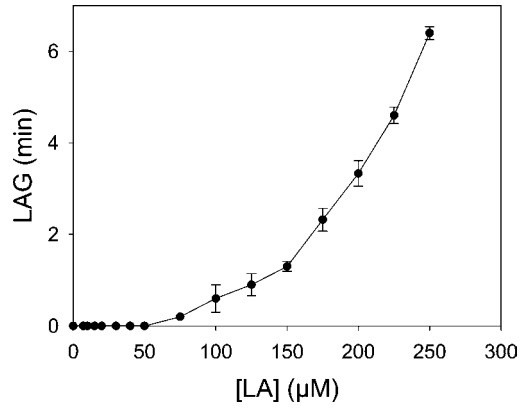


Figure 2. Effect of the substrate concentration on the duration of the lag period. The reaction medium contained different amounts of LA and 0.01 unit of LOX in 0.1 M sodium borate buffer at pH 10.0.

Lag Phase. The reaction catalyzed by *T. claveryi* LOX exhibited an induction period that was longer than that observed for LOX-1 from soybeans (*18*) and could therefore be studied without using stopped-flow techniques. We studied the effect of different factors (substrate, enzyme, and 13- and 9-HPOD concentrations) on the oxidation rate of linoleic acid by *T. claveryi* LOX at pH 10 and on the duration of the lag period.

Effect of Substrate Concentration. When the duration of the induction period was measured at different concentrations of the substrate, it was seen to increase with the concentration of LA (**Figure 2**). These results were explained by the model proposed by Schilstra and co-workers (*17*, *18*) for soybean LOX-1 (**Scheme 1**). According to this model, both the substrate and the reaction product compete for the same binding site on Fe^{2+}-LOX (steps H and A of **Scheme 1**) and Fe^{3+}-LOX (steps C and F of **Scheme 1**), and the lag period is due to a high substrate/product ratio, which results in a low concentration of active enzyme. When the product (LOOH) binds to Fe^{2+}-LOX (steps A and B of **Scheme 1**), this species is converted into the

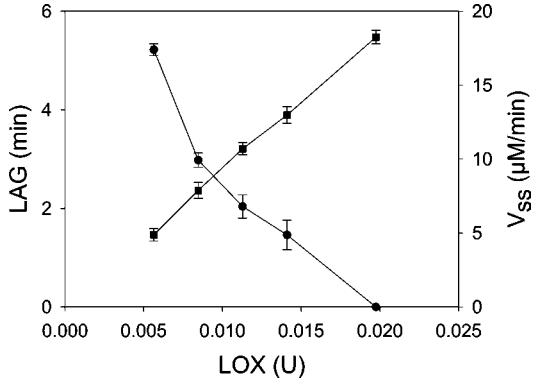


Figure 3. Effect of the enzyme concentration on the duration of the lag period (●) and on the steady-state rate (■). The reaction medium contained 180 μM LA and different amounts of *T.* claveryi LOX in 0.1 M sodium borate buffer at pH 10.0.

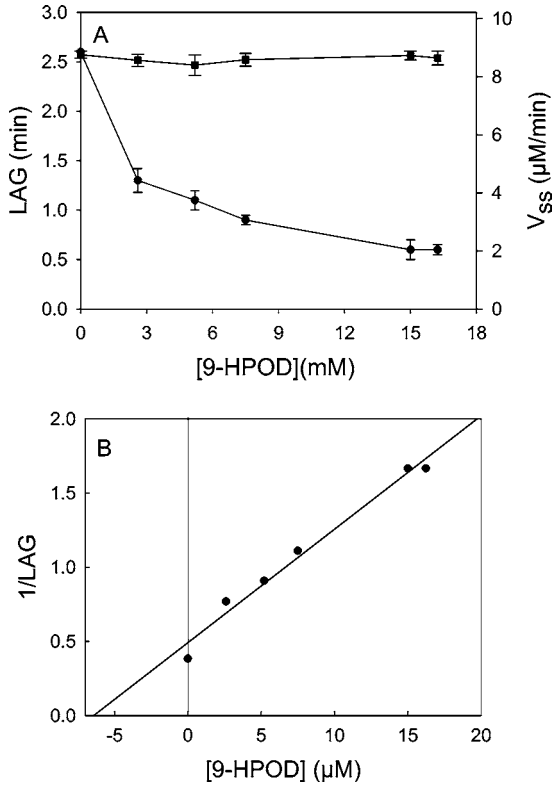


Figure 4. (A) Effect of 9-HPOD on the duration of the lag period (●) and on the steady-state rate (■). The reaction medium consist of 180 μM LA, different concentrations of 9-HPOD, and 0.01 unit of LOX in sodium borate buffer at pH 10.0. (B) Determination of the activation constant (K_{act}) for 9-HPOD (see the Material and Methods for details).

active form, Fe^{3+}-LOX. However, the binding of the substrate (LH) to Fe^{2+}-LOX leads to a dead-end complex (step H of **Scheme 1**). Thus, an increase in the substrate concentration initially displaces the equilibrium toward the formation of a dead-end complex, increasing the length of the lag period. LOOH increases with the progress of the reaction, and thereafter, the enzyme returns to the catalytic cycle.

Effect of Enzyme Concentration. To study the duration of the induction period as a function of the enzyme concentration, the progress curves at a starting substrate concentration of 180 μM were recorded for a range of LOX concentrations (0.005−0.02 units). The duration of the lag was inversely proportional to the enzyme concentration (**Figure 3**). This reciprocal relationship between the lag period and the enzyme concentration has

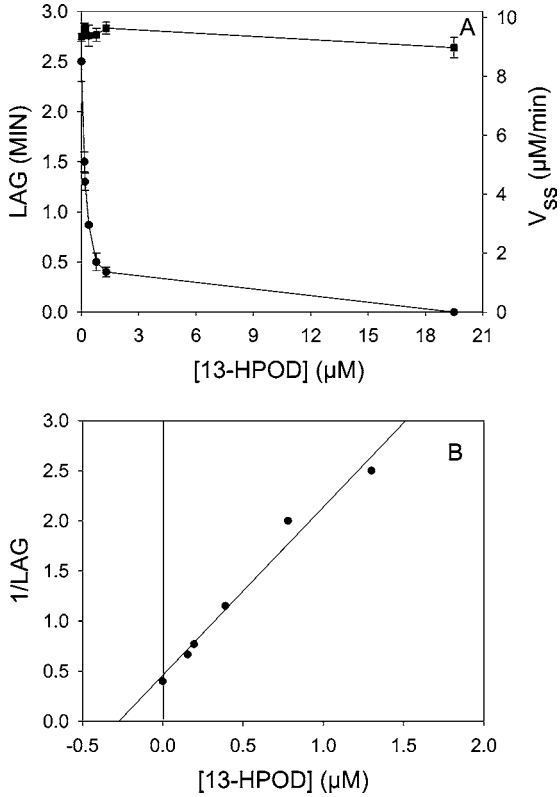


Figure 5. (A) Effect of 13-HPOD on the duration of the lag period (●) and on the steady state-rate (■). The reaction medium consist of 180 μM LA, different concentrations of 13-HPOD, and 0.01 unit of LOX in sodium borate buffer at pH 10.0. (B) Determination of the activation constant (K_{act}) for 13-HPOD (see the Material and Methods for details).

been described for the oxygenation of different fatty acids by soybean and tomato LOXs (*18*, *34*, *39*). The lag period was completely eliminated using 0.02 unit of LOX. The steady-state rate was linearly dependent on the enzyme concentration (**Figure 3**).

Effect of 13- and 9-HPOD. As stated in the Introduction, the two-step mechanism (*16*−*19*) assumed that the hydrogen abstraction from the substrate (LH) to form the radical L* can only be catalyzed by Fe^{3+}-LOX (steps C and D of **Scheme 1**). The lag phase of this reaction corresponded to the time needed to transform the inactive Fe^{2+}-LOX into the active Fe^{3+}-LOX, with this oxidation step being caused by the hydroperoxide product (step A of **Scheme 1**). This model also explained the results obtained with *T. claveryi* LOX. The presence of micromolar amounts of fatty acid hydroperoxides at the start of the reaction catalyzed by this enzyme caused a reduction and even the disappearance of the induction period (part **A** of **Figures 4** and **5**) but did not affect the steady-state rate. Both 13- and 9-HPOD were able to diminish the length of the lag phase, although 13-HPOD, the product of the reaction catalyzed by LOX from *T. claveryi*, seemed to be more effective than 9-HPOD. In this paper, we propose a method to quantify the effectiveness of different hydroperoxides as LOX activators by calculating their activation constants (K_{act}). With L being the lag time in the absence of HPOD and λ being the lag time in the presence of HPOD, a plot of $(L - \lambda)$ versus the initial [HPOD] gave a hyperbolic curve, which approached L as an asymptote when HPOD increased and λ became small. This curve was analogous to a plot of velocity versus the substrate concentration. Because it was difficult to estimate K_{act} reliably from this hyperbolic curve, another way of treating the data is to plot $(1/\lambda)$ versus initial [HPOD] (part **B** of **Figures 4** and **5**)

and to calculate the activation constant using the following equation (*40*):

$$1/\lambda = 1/L + (1/L[\text{HPOD}]/K_{act}) \quad (3)$$

The values obtained were 0.3 μM for 13-HPOD and 6.4 μM for 9-HPOD. Thus, 13-HPOD is ~21-fold more effective than 9-HPOD as a *T. claveryi* LOX activator.

CONCLUSION

The effectiveness of different hydroperoxides as activators of LOX can be quantified for the first time using the method proposed in this paper. The calculation of the activation constants indicates that 13-HPOD (the product of *T. claveryi* LOX with LA) is ~21-fold more effective than 9-HPOD as an activator of this enzyme. In addition, the results obtained indicate that LOX from *T. claveryi* shares various kinetic features with soybean LOX-1, which can be explained using the model proposed by Schilstra and co-workers *(17−19*) for the latter enzyme.

ABBREVIATIONS USED

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CD, cyclodextrin; cmc, critical micellar concentration; HPOD, hydroperoxy octadecadienoic acid; LA, linoleic acid; LA_f, monomeric linoleic acid; LH, fatty acid substrate; LOOH, fatty acid hydroperoxide; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; PUFA, polyunsaturated fatty acid.

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