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Effect of exogenous iron on aerobic catalytic mechanism of soybean lipoxygenase

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Abstract

Effect of exogenous iron on the enzymatic activity of soybean lipoxygenase (LOX) in aerobic catalysis is investigated in this paper. It is found that a certain level of exogenous Fe^{3+}, Fe^{2+} or linoleic acid hydroperoxide (LA-HPOD) could speed up the oxidation process of linoleic acid catalyzed by soybean LOX. Absorbency band between 300 and 400 nm in UV spectrum of soybean LOX was strengthened once upon adding of Fe^{3+} but only changed slightly with adding of Fe^{2+} or other ions. Therefore, Fe^{3+} is much more affinitive to the catalytic site of LOX than other ions, although the previous experimental results have indicated that the iron inside native enzyme mainly exists as a more stable Fe^{2+}-LOX state. The potential function of exogenous Fe^{3+}, Fe^{2+} or LA-HPOD in aerobic catalysis mechanism of soybean LOX is proposed as electron transfer from the intrinsic electron donor to the extrinsic electron acceptor, which speeds up the conversion from Fe^{2+} to Fe^{3+} in situ inside the active site of LOX. Since the above exogenous matters can accelerate the initial rate and thus the overall rate of aerobic catalysis of linoleic acid catalyzed by LOX, it becomes evident from our study that the conversion from Fe^{2+} to Fe^{3+} in situ is the control step in the above aerobic reaction.

Keywords: Lipoxygenase; Iron; Mechanism; Dynamics

1. Introduction

Lipoxygenase (LOX) is widely distributed in all plants and catalyzes the oxidation of polyunsaturated fatty acids containing the *cis,cis*-1,4-pentadiene moiety to the corresponding hydroperoxides (HPOD) of conjugated dienes. LOXs are monomeric and contain one nonheme, nonsulfur iron atom per molecule, which is thought to be essential for their catalytic behaviors [1–4]. Soybean LOX-1, used to be considered as a model, has been the most thoroughly characterized LOX. The iron in LOX-1 is generally believed to be in an octahedral ligand environment [5,6], whose properties and microenvironment have been most intensely examined.

The role of the iron has become the focus of recent studies on the catalytic mechanism of LOX [7–11]. The iron plays a redox role in the catalyzed reaction, with the formation of Fe^{3+}-LOX it accepts an electron from the specific 3-methylene of 1,4-pentadiene moiety in the polyunsaturated fatty acid. LOX-1 was isolated from soybean seeds, which referred to as the native enzyme mainly contains Fe^{2+} in a nonheme environment. Fe^{2+}-LOX is essentially colorless and shows no EPR spectroscopic features. Treatment of Fe^{2+}-LOX with equimolar hydroperoxide results in oxidation of the cofactor to Fe^{3+}, the latter has been thought as the active form to catalyze aerobic oxidation. It is this form of the enzyme that has a characteristic pale yellow color and an EPR signal in the g-6 region, which can be reversibly reduced to the Fe^{2+}-LOX upon addition of some substrate such as linoleic acid to the enzyme under anaerobic conditions [1,12].

Fig. 1 shows a documentally proposed scheme of the aerobic catalysis of LOX [2]. The active LOX-1 functions by a redox cycle between high-spin Fe^{3+} and high-spin Fe^{2+} as fueled by substrate and fatty acid peroxy radical. The Fe^{3+}-LOX

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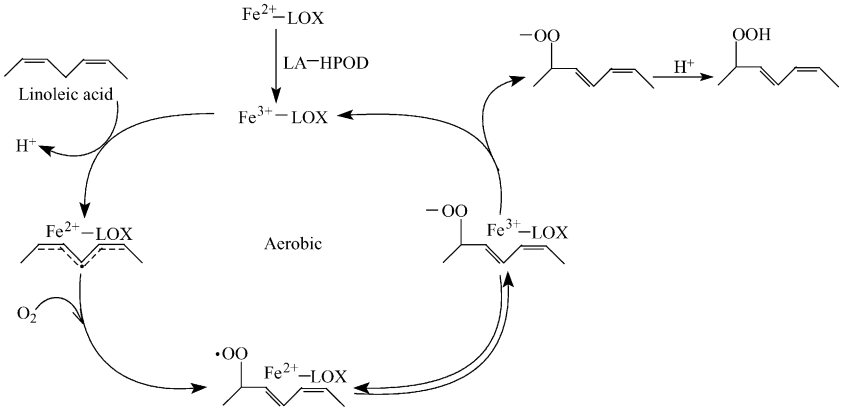

Fig. 1. Scheme of aerobic oxidation of linoleic acid by LOX. Fatty acid structures are abbreviated to show only C-8 to C-14 [2].

is responsible for oxidizing *cis,cis*-1,4-pentadiene moiety of the fatty acid to the pentadienyl radical, and the latter reduces Fe^{3+} to Fe^{2+} in turn.

Kinetic models for LOX catalysis fall into two classes [12,13]. In the fist model, the inherent Fe^{2+} form of the enzyme is considered catalytically inactive. Full catalytic activity of the enzyme is acquired only when the iron cofactor of the enzyme is oxidized from the state Fe^{2+} to Fe^{3+} by HPOD [14,15]. In another model, Fe^{2+}- and Fe^{3+}-LOX are thought both catalytically active but in different levels. Although both of these models have been independently supported by a variety of experimental data, no convincing evidence has yet been presented in favor of one model versus the other.

Importance of intrinsic iron inside of LOX to its aerobic catalysis has been widely studied. But further research about the function of exogenous iron in the mechanism of LOX catalysis has been scarcely reported [16]. When we investigate the LOX aerobic catalytic reaction using different stirrers, i.e. PTFE coated iron stirrer, stainless steel stirrer and iron stirrer, it is found that the one with iron stirrer proceeded at a highest rate, while that with PTFE coated iron stirrer induced the lowest rate. This phenomenon suggests that the micro-iron on the surface of stirrer can stimulate the LOX catalytic oxidation. In this report, we would like to discuss the state of iron in the native lipoxygenase, the effect of exogenous iron (including Fe^{2+} and Fe^{3+}) and conversion rate from Fe^{2+}-LOX to Fe^{3+}-LOX on the LOX activity in aerobic catalysis. Supplemental aerobic enzymatic mechanism of LOX and the external factors that can affect the enzyme activity are further elucidated.

2. Experimental

2.1. Chemicals

Soybean LOX was purchased from Sigma (70,600 U/mg). Linoleic acid (LA, 98% of purity) was purchased from Shanghai Chemicals Co., China. Linoleic acid hydroperoxide (LA-HPOD) was synthesized in our laboratory. All other reagents are of analytical grade.

2.2. Enzymatic oxidation

In a typical procedure, a given amount of linoleic acid (7.8 or 100 mM) was dissolved in 20 ml of borate buffer (0.2 M, pH 9.0), and air at atmospheric pressure was used as oxygen source. The reaction was started upon the addition of a given dosage of enzyme at room temperature or 10 °C. No antifoam reagent was added. After 2 h, the reaction was terminated by addition of 2 M H_2SO_4 to pH 3.5. Extracted twice with ether, the organic phases were combined and washed with water, then dried over sodium sulfate. The ether was vaporized under N_2 stream and the LA-HPOD oily liquid residue was obtained.

2.3. Analysis

Lipoxygenase activity was determined by measuring the increase in absorbance at 234 nm with a spectrophotometer (TU-1901 UV spectrophotometer) according to [17].

Duplicate samples (40 μl for low LA concentration (7.8 mM) and 5 μl for high LA concentration (100 mM)) were taken from the reaction system and diluted with 5 ml ethanol to stop the reaction and the absorbance was measured at 234 nm. The reaction was terminated when the absorbance became steady.

The amount of Fe^{3+}-LOX in the solution is linearly related to the absorbance at 330–400 nm [12,13]. The absorbance at 360 nm, a region attributable to an iron combination was measured when extrinsic iron was added to LOX solution.

3. Results and discussion

3.1. Role and inherent state of iron in lipoxygenase

Iron is the indispensable part in majority of lipoxygenase. The enzyme activity would be lost if iron were removed from

the enzyme [18]. The original demonstration that lipoxygenases contain iron was based on the extraction of the metal by *o*-phenanthroline in the presence of reducing agents. Reconstitution of the apoenzyme was not achieved under a variety of conditions. In our experiments, *o*-phenanthroline was used to take iron away from the native enzyme, no obvious change of enzyme activity could be found in the beginning once upon *o*-phenanthroline was added to the enzyme solution under aerobic conditions at room temperature. Whereas there observed a 30% loss of enzyme activity after 36 h. The iron-phenanthroline complex could be separated from the protein by dialysis. Our experimental results indicated that the subsequent direct addition of excess iron, i.e. ferric ammonium sulfate, to the enzyme solution (in 0.2 M, pH 9.0 borate buffer) would not restore enzyme activity under aerobic conditions at room temperature. But Kariapper and co-workers reported the discovery of conditions for successful extraction of iron from soybean LOX-3 with Chelex-100 (a resin of sodium form) and reconstitution of catalytic activity from the soluble apoenzyme with ferric ammonium sulfate in sodium bicarbonate buffer. They reported that LOX activity was restored from 1 to 90 ± 10% of the original activity [19]. Thus we think, for LOX-1, the reconstitution of catalytic activity from apoenzyme may be feasible only when the appropriate conditions were found.

Fe^{2+}-LOX has been stated in literatures to be the main form of LOX in native state. Effects of exogenous factors on UV spectrum of LOX were studied here to determine whether the structure of LOX could be affected. When the enzyme solution was titrated with Fe^{3+} a series of UV spectrum in 0.2 M, pH 9.0 borate buffer were recorded. Two phenomena could be observed from the recorded UV spectrum. Firstly, the absorbency band between 300 and 400 nm was strengthened when Fe^{3+} increased (Fig. 2a), which showed that there was an interaction between the exogenous Fe^{3+} and the enzyme. Secondly, the 360 nm absorbency touch the top and then remained constant when exogenous Fe^{3+} increased (Fig. 2b), which implied that more Fe^{3+}-LOX might have been formed, i.e. intrinsic Fe^{2+} was converted to Fe^{3+} in situ in the active center inside of LOX. It is therefore reasonable to believe that electron transfer between exogenous Fe^{3+} and intrinsic Fe^{2+}, in which the latter acts as the electron donor, causes these phenomena and the former performs as the electron acceptor.

Similarly, when enzyme solution was titrated with Fe^{2+}, absorbance at 360 nm increased little (Fig. 2b), which indicated that interaction between exogenous Fe^{2+} and LOX was much weaker than that in the Fe^{3+} case. A plateau was observed in Fig. 2b, which could be explained as follows: exogenous Fe^{2+} in LOX solution was more easily oxidized to Fe^{3+} compared with intrinsic Fe^{2+} and then electron transfer effect occurred between the intrinsic Fe^{2+} and the neonatal Fe^{3+}. While other ions including calcium and sodium ions had no effect on the UV spectrum of LOX at 360 nm (Fig. 2b).

As shown in Fig. 3, when the native enzyme was titrated directly with LA-HPOD, the absorbance at 360 nm also increased. But a plateau was reached when about 1.2 μmol of LA-HPOD had been added into the 3.5 ml of lipoxygenase solution. As mentioned before, the pre-oxidized product LA-HPOD could specifically oxidize Fe^{2+}-LOX, which led to the formation of Fe^{3+}-LOX. This phenomenon can also be successfully explained by the above electron transfer effect because in this case the exogenous LA-HPOD replaces the role of the exogenous Fe^{3+} as the electron acceptor. When the conversion of Fe^{2+}-LOX to Fe^{3+}-LOX approached equilibrium, no more increase in absorbance at 360 nm was observed with ever-increasing amount of LA-HPOD.

The above experimental results indicated that the iron inside of native enzyme mainly exists as Fe^{2+} state. Exogenous Fe^{3+}, Fe^{2+} or LA-HPOD leads more Fe^{3+}-LOX quickly formed from Fe^{2+}-LOX by virtue of the electron transfer. But the influence of exogenous Fe^{2+} on UV spectrum of LOX is much weaker than both exogenous Fe^{3+} and LA-HPOD.

3.2. Effect of exogenous LA-HPOD on LOX catalysis

Based on the above result, LA-HPOD has influence on the conversion from Fe^{2+}-LOX to Fe^{3+}-LOX; it is therefore necessary to further study the possible effect of LA-HPOD on LOX catalysis. Fig. 4 shows the effect of exogenous

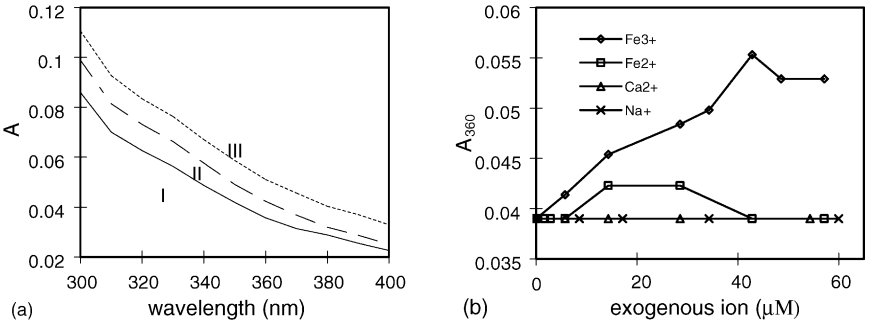

Fig. 2. Effect of exogenous Fe^{3+} and other ions on the spectrum of LOX. (a) Spectrum of LOX incubated with exogenous Fe^{3+} under aerobic conditions. (I) Native lipoxygenase in borate buffer (0.2 M, pH 9.0) at room temperature. (II) Conditions as for curve a, but with 22.8 μM of Fe^{3+}. (III) Conditions as for curve a, but with 42.8 μM of Fe^{3+}. (b) Effect of exogenous ions on the absorbance of lipoxygenase at 360 nm. Native lipoxygenase in borate buffer (0.2 M, pH 9.0) at room temperature.

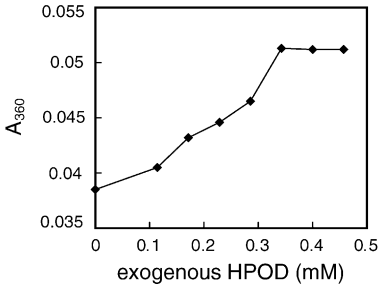

Fig. 3. Effect of exogenous LA-HPOD on the absorbance of lipoxygenase at 360 nm. Native lipoxygenase in borate buffer (0.2 M, pH 9.0) at room temperature.

LA-HPOD level on the LOX catalyzed oxidation. Addition of LA-HPOD abolished the lag phase in the hydroperoxidation of linoleic acid, which implied a direct interaction between LA-HPOD and the Fe^{2+}-LOX. Thus Fe^{2+}-LOX was quickly converted to Fe^{3+}-LOX with the incubation of exogenous LA-HPOD. When exogenous LA-HPOD level in the reaction mixture being 0.8 nM, the highest reaction rate was reached. Furthermore, excessive exogenous LA-HPOD had a negative effect on the reaction, maybe due to the product inhibition to the enzyme.

3.3. *Effect of exogenous Fe^{3+} on LOX activity and catalysis*

Fe^{3+}-LOX has been proven to be the active form of LOX while Fe^{2+}-LOX is the main form in native state. Initiation of LOX catalysis can be divided into two steps, i.e. Fe^{2+} inside of LOX catalytic site is oxidized to Fe^{3+} and then Fe^{3+}-LOX combines with linoleic acid. The time course for converting substrate to product is much shorter than that for the conversion of Fe^{2+}-LOX to Fe^{3+}-LOX because the latter process involves a pronounced lag phase [13]. Effect of exogenous Fe^{3+} on LOX catalysis was explored in this paper to illustrate whether the process converting Fe^{2+}-LOX to Fe^{3+}-LOX is the control step. Optimizing this step may abolish the lag

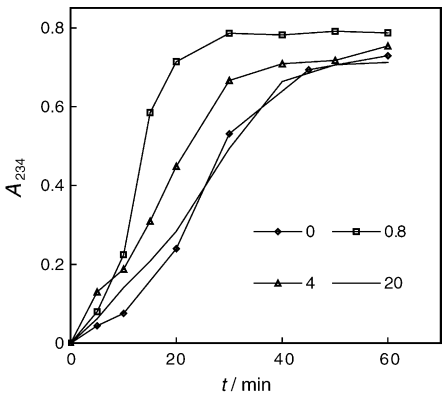

Fig. 4. Effect of exogenous LA-HPOD on LOX catalysis. LA of 7.8 mM, 0–20 nM of exogenous LA-HPOD, 10 °C and pH 9.0.

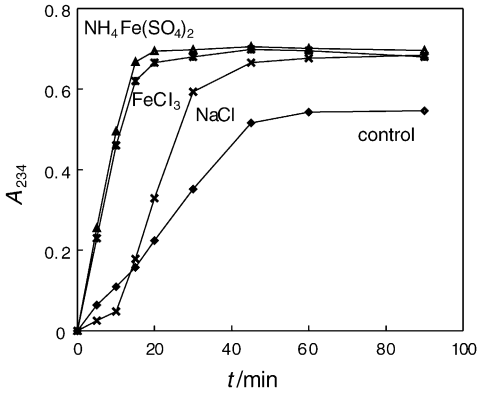

Fig. 5. Effect of exogenous ions on LOX catalysis. LA of 7.8 mM in 20 ml of pH 9.0 borate buffer at 10 °C, with 0.5 μM of $NH_4Fe(SO_4)_2$ or $FeCl_3$ but 1.5 μM of NaCl.

phase in the initiation state consequently accelerates overall speed of LOX catalysis.

Under the same conditions, ferric ammonium sulfate, sodium chloride or ferric chloride was added to the reaction, and the results are shown in Fig. 5. With same iron level (0.5 μM), ferric ammonium sulfate and ferric chloride promoted the reaction markedly. With same chloride anion level (1.5 μM), the rate increment with ferric chloride was higher than that with sodium chloride. So, it was Fe^{3+} that played an essential role in the increase of reaction rate. As shown in Fig. 5, the initial rate of enzymatic reaction with exogenous Fe^{3+} was markedly higher compared to that without exogenous Fe^{3+} and the time to reach reaction equilibrium was shortened obviously. This indicated that the total activity of LOX was increased when Fe^{3+} was added. It is thought that the LOX catalysis is initiated by the binding of appropriate unsaturated substrates at active catalytic site [13], so exogenous Fe^{3+} accelerated the initiation state because it shortened the activation course of LOX by electron transfer. This agreed to the fact of exogenous Fe^{3+} effect on the UV spectrum of LOX (Fig. 2b).

As for enhancement of reaction rate by sodium chloride (see Fig. 5), it may be explained as follows: ions occupies the inhibitory binding site of enzyme for substrate, which makes the concentration of substrate relative to the active site enhanced [20]. This can be called as salt effects, which is not fit for the explanation for exogenous iron effect.

Fig. 6 shows effect of exogenous Fe^{3+} on LOX catalytic reactions under low (7.8 mM) (Fig. 6a) and high (100 mM) (Fig. 6b) LA levels. The initial rate was improved to some extent in both cases. At low LA level, enhancement of reaction rate was more notable than that at high LA level. When exogenous Fe^{3+} in the reaction mixture reached 0.5 μM, the curves started with a burst vertical line instead of a lag phase and the highest reaction rate reached. Further increasing the amount of Fe^{3+}, the effect of exogenous Fe^{3+} became inconspicuous. It shows that excessive Fe^{3+} is not still beneficial for the enzymatic reaction and there is an optimal level of exogenous Fe^{3+} for LOX catalysis (Fig. 6a). We think this is because

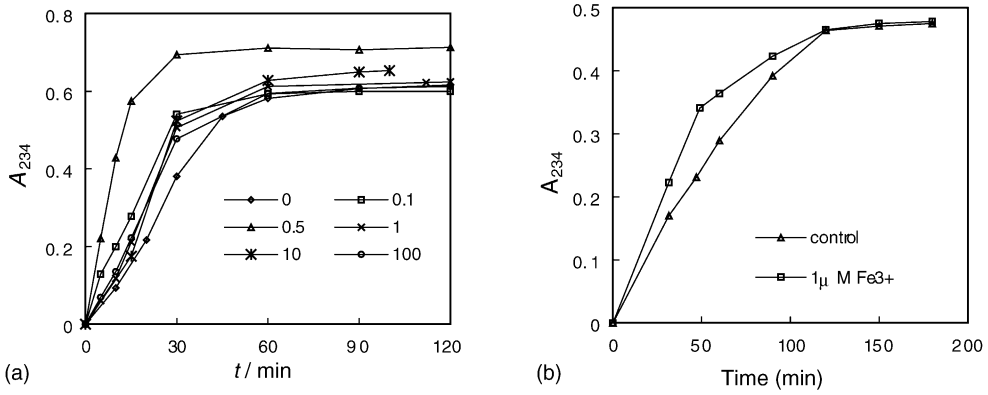

Fig. 6. Effect of exogenous Fe^{3+} on LOX catalysis. (a) Lower LA concentration (7.8 mM) 5×10^5 U/l of LOX, 0–100 μm of exogenous Fe^{3+} under pH 9.0 at 10 °C. (b) Higher LA concentration (100 mM) 10^6 U/l of LOX, 1 μm of exogenous Fe^{3+} under pH 9.0 at 10 °C

excessive exogenous Fe^{3+} will also damage the protein structure besides the above promotion effect. In our experiment the positive effect of Fe^{3+} was dominant and no obvious inhibition was observed with exogenous Fe^{3+}. Fig. 6b showed at LA concentration of 100 mM the inhibition of substrate became more obvious although the ratio of LOX/exogenous Fe^{3+} was keeping with the optimal ratio (10^6 U/μM) in Fig. 6a. Thus exogenous Fe^{3+} effect was negligible due to the competition between the substrate inhibition and Fe^{3+} promotion.

Accelerating the conversion of Fe^{2+}-LOX to Fe^{3+}-LOX can affect the dynamic course by reducing the lag phase of initial reaction and accelerate the total reaction rate. Exogenous Fe^{3+} accepts the electron to induce the conversion from Fe^{2+} to Fe^{3+} in situ inside of the active center of LOX. Therefore, addition of an optimal amount of Fe^{3+}, as well as LA-HPOD, into the reaction system can clearly promote the enzymatic reaction, especially for low LA level.

3.4. Effect of exogenous Fe^{2+} on LOX catalysis

As mentioned above, suitable amounts of exogenous Fe^{3+} can enhance the LOX catalysis markedly. It is curious if exogenous Fe^{2+} has the same effect. When the concentration of ferrous ammonium sulfate in reaction mixture reached 5 nM, the enzymatic reaction rate increased markedly as shown in Fig. 7. In addition, the lag phase was abolished, but when the exogenous Fe^{2+} concentration was higher than 5 nM, the inhibition in initial reaction occurred. The inhibition became more obvious with ever-increasing exogenous Fe^{2+} concentration. The above results can be explained as follows: (1) Addition of a little exogenous Fe^{2+} into the reaction system promotes oxidation from Fe^{2+} to Fe^{3+} inside of LOX, which accelerates the initial reaction rate. Consumption of LA-HPOD by Fe^{2+} was negligible in this case. (2) Exogenous Fe^{2+} of 5 nM was a fit concentration to abolish the lag phase (Fig. 6b). (3) Excessive exogenous Fe^{2+} will consume more LA-HPOD product in the system due to oxidation to result in total rate inhibition although there is still a positive effect of converting the exogenous Fe^{2+} to Fe^{3+} simultaneously. In our experiments, 2.5 μM of exogenous Fe^{2+} made the catalysis rate slow down whereas the concentration of Fe^{2+} between 5 nM and 2.5 μM did not affect LOX catalysis obviously. Thus, exogenous Fe^{2+} would promote the LOX catalysis if an appropriate amount were used.

3.5. LOX catalytic mechanism and its control factor

In our experiments, some external factors affected the structure and the activity of LOX in aerobic catalysis have been found. Further explanation to the aerobic catalytic mechanism of soybean LOX and the external regulation mechanism to some extent were proposed as shown in Fig. 8. Exogenous Fe^{3+} has an effect on the spectrum of LOX between 300 and 400 nm and induces the conversion of Fe^{2+}-LOX to Fe^{3+}-LOX and then abolishes the lag phase of catalysis as well as LA HPOD. Exogenous Fe^{2+} has a little effect on the UV spectrum of LOX between 300 and 400 nm. But suitable exogenous Fe^{2+} can promote the reaction rate clearly. Electron transfer is the dominating factor during above process. We considered that any factor that can promote the conversion from Fe^{2+}-LOX to Fe^{3+}-LOX would abolish the lag phase and accelerate the reaction rate. These results provide a somewhat clearer image to promote the LOX aerobic enzymatic reaction, which is illustrated in Fig. 8.

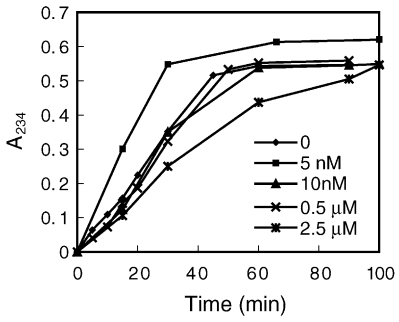

Fig. 7. Effect of exogenous Fe^{2+} on LOX catalysis. LA of 7.8 mM, 5 nmol/l–2.5 μM of $(NH_4)_2Fe(SO_4)_2$ under pH 9.0 at 10 °C.

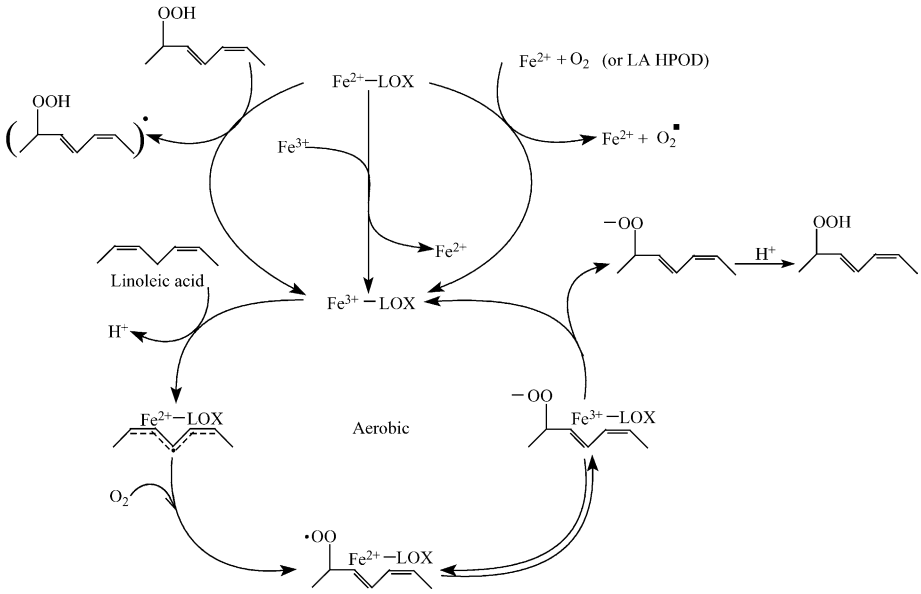

Fig. 8. Modified scheme of aerobic oxidation of linoleic acid by LOX. Fatty acid structures are abbreviated to show only C-8 to C-14.

4. Conclusion

Based on the above investigations mainly with UV analysis the effect of exogenous iron on LOX aerobic catalytic reaction was examined. Suitable amount of exogenous Fe^{3+} and Fe^{2+} can shorten the lag phase of oxidation thus enhance the reaction rate as well as LA-HPOD. Addition of Fe^{3+} makes the absorbance band between 300 and 400 nm in UV spectrum of lipoxygenase strengthened but absorbance changed little with Fe^{2+}. The possible mechanism of aerobic catalysis of LOX and the effect of exogenous Fe^{3+} on catalytic mechanism was proposed as electron transfer effect. Any factor that can promote the conversion from Fe^{2+}-LOX to Fe^{3+}-LOX will be considered to abolish the lag phase and accelerate the reaction rate.

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