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Mechanistic studies on the effect of veratryl alcohol on the lignin peroxidase catalyzed oxidation of pyrogallol red in reversed micelles

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Abstract: The lignin peroxidase (LiP) catalyzed oxidation of pyrogallol red (PR) in the absence and presence of veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA) was carried out in bis (2-ethylhexyl) sulfosuccinate sodium (AOT)/ polyoxyethylene lauryl ether (Brij30) reversed micelles to elucidate the role of VA. Results indicated that VA could accelerate the LiP catalyzed oxidation of PR, especially at low H_2O_2 concentrations. Unlike in bulk aqueous medium, the protection of LiP by VA in the present medium was relatively unsubstantial, even at high H_2O_2 concentrations. Analysis of data from a series of experiments showed that the enhancement of the PR oxidation caused by VA was mainly due to the indirect oxidation of PR by $VA^{+\bullet}$ from the LiP catalyzed oxidation of VA. It was also found that at the same protector concentration (40 μM), VA (the physiological substrate of LiP) was less effective than PR (a phenolic compound) in protecting LiP from the H_2O_2 derived inactivation. This novel phenomenon deserves further study.

Keywords: Lignin peroxidase, reversed micelle, veratryl alcohol, pyrogallol red, mediated oxidation

1 Introduction

The use of white rot fungi, such as *Phanerochaete chrysosporium*, in the degradation of environmental pollutants is a hot topic in the field of environmental science and engi-

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neering [1–7]. Their ability to degrade aromatic pollutants comes from the extracellular enzymes produced under ligninolytic conditions, such as lignin peroxidase (LiP) [8–17]. The LiP catalyzed oxidative degradation of the aromatic pollutants is correlated with the one-electron oxidation mechanism of LiP as well as with the similarity in structure unit between the aromatic compounds and lignin.

LiP is a water soluble protein, so the efficiency of the degradation depends on, to some extent, the bioavailability of the aromatic compounds in water. Most aromatic pollutants, however, are less soluble in aqueous solution. In less polar organic solvents the solubility of these hydrophobic compounds is usually increased, but in pure organic solvent the native enzyme does not have its full activity. So such a medium is required in which not only these hydrophobic compounds could be concentrated but also the enzyme could retain its activity.

A reversed micelle is a good choice as medium. It is composed of oil, water, surfactant and/or cosurfactant. Because of its peculiar microenvironment, enzymes hosted in such medium are usually catalytically active [18, 19]. In reversed micellar medium the degradation efficiency could be greatly improved due to high substrate concentration and high enzyme dispersion. Studies on the catalytic performance of LiP in reversed micellar medium have been reported [20, 21]. Recently we have found that LiP exhibits high activity in a mixed reversed micellar system–bis (2-ethylhexyl) sulfosuccinate sodium (AOT)/polyoxyethylene lauryl ether (Brij30) mixed reversed micelles [22].

Veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA), a secondary metabolite of *Phanerochaete chrysosporium*, is known to play an important role in the oxidative biodegradation of lignin [10, 23, 24]. Studies in aqueous solution [9, 10, 14, 24–28] indicated that addition of VA could greatly enhance the degradation efficiency of aromatic compound catalyzed by LiP. How does VA work in a reversed micellar medium? Exploration of the effect of VA in reversed micellar medium will be helpful for us to improve the degradation efficiency of aromatic compound catalyzed by LiP. Moreover, the exploration is conducive to clarifying the real role of VA during the natural degradation of lignin by the fungus because the degradation was carried out in a colloidal medium which is very similar to reversed micelles but quite different from bulk aqueous medium.

To facilitate the present study, a water-less-soluble triphenylmethane dye, pyrogallol red (PR), was chosen as substrate and the mixed AOT/Brij30/isooctane/water reversed micelle as medium. The LiP catalyzed oxidation of PR in the absence or presence of VA was carried out in the medium to elucidate the role of VA. The present results indicated that VA could accelerate the LiP catalyzed oxidation of PR, especially at low H_2O_2 concentrations. Unlike in bulk aqueous medium, the protection of LiP by VA in the present medium was less by comparison, even at high H_2O_2 concentrations. To elucidate the roles of VA, a series of experiments was carried out. For example to shed light on the indirect oxidation of PR by veratryl alcohol cation radical ($VA^{+\bullet}$) and to eliminate the inactivation of LiP by excess H_2O_2, an enzymatic H_2O_2 supply strategy was used to replace the external H_2O_2 supply strategy. Analysis of data showed that the enhancement of the PR oxidation caused by VA was mainly due to the indirect oxidation of PR by

$VA^{+\bullet}$ from the LiP catalyzed oxidation of VA. During the study, we also found that at low concentrations, VA was less effective than PR in protecting LiP from the H_2O_2 derived inactivation although PR is a phenolic compound. This was a novel phenomenon which has not been reported before and studies on the role of PR in protecting LiP are under way.

2 Materials and methods

2.1 Chemicals

Veratryl alcohol (VA) was purchased form Aldrich Co., USA. AOT and Brij30 were purchased from Sigma Co., USA. Glucose oxidase (GOD) was obtained from Fluka Co., Switzerland. All other chemicals (analytical grade) such as pyrogallol red (PR), H_2O_2 *et al.*, were obtained from China. Triply distilled water was used throughout the experiments.

2.2 Preparation of the mixed reversed micellar medium

The mixed AOT/Brij30 reversed micellar medium ([AOT] + [Brij30] = 200 mM) was prepared by first dissolving appropriate amount of AOT and Brij30 in isooctane ([AOT]= 93 mM, [Brij30] = 107 mM) and, then, adding an aliquot of citric buffer (0.1 M, pH= 4.8) to the mixture (the molar ratio of water to the surfactants (ω_0) was set at 23). A clear solution was soon obtained.

2.3 Lignin peroxidase

The LiP from *Phanerochaete chrysosporium* F. F. Lombard ME446 (ATCC 34541) was isolated and purified according to the procedure reported in our previous paper [29]. The concentration of LiP was measured using a molar absorptivity of 169 $mM^{-1}cm^{-1}$ at 409 nm [10].

2.4 Measurement of the initial rate of the LiP catalyzed oxidation of VA or PR

For VA oxidation: A stock solution of VA (20 mM) in isooctane containing 200 mM AOT+Brij30 (the molar percentage of Brij30 (χ_B) was 0.53) was prepared by dissolving an appropriate amount of VA in the AOT/Brij30 solution in isooctane. According to the final concentration of VA in the reaction system, the volume (x mL) of the needed VA stock solution was calculated and transferred, together with y mL solution of the AOT/Brij30 in isooctane (200 mM, χ_B=0.53), to a 1 cm long quartz cuvette ($x+y=3$), then an aliquot of citric buffer (0.1 M, pH 4.8) and an aliquot of the LiP and GOD (0.2 $mg{\cdot}mL^{-1}$, added when glucose was used to initiate the reaction) aqueous solutions

were added into the cuvette. The resulting solution was mixed thoroughly. The oxidation reaction was initiated by adding an aliquot of H_2O_2 (10 mM) or glucose aqueous solution (100 mM, prepared with 0.1 M citrate buffer, pH 4.8) ($\omega_0 = 23$). After quick mixing, a plot of absorbance (A) at 310 nm versus time (t) was recorded. From the slope of the linear portion of the $A_{310} - t$ curve, the initial rate of the LiP catalyzed oxidation of VA could be calculated. The molar extinction coefficient of veratraldehyde at 310 nm was 9300 $M^{-1}cm^{-1}$ [20, 26].

For PR oxidation: Steps used for the measurement of the initial rate of the LiP catalyzed oxidation of PR were the same as for that of VA except that the stock solution of PR was 80 μM and the monitoring wavelength was 500 nm (the characteristic peak of PR), and that the initial rate was expressed as $-dA/dt$ in absorbance units $\times$ min^{-1}.

All concentration values given in the figure legends were the final concentrations of experimental systems and that unless specified, all experiments were carried out in the AOT/Brij30 mixed reversed micellar medium at 30 °C.

2.5 Kinetic spectrum of the LiP catalyzed oxidation of PR

The reaction system used here was the same as that in Section 2.4 except that, prior to the initiation of the reaction by H_2O_2 or glucose, a reagent blank of the corresponding system was used as reference.

In the kinetic spectra, the first curve (from top to bottom) was recorded prior to the initiation, the second one was recorded immediately after the initiation, and from then on, scanning was carried out at a given time interval (details see the figure legend). The term $\Delta A/\Delta t$ in the text denoted an increment in absorbance in one minute and dA/dt denoted the initial rate (absorbance units $\times$ min^{-1}).

2.6 Effects of VA and PR on the stability of LiP in the presence of H_2O_2

First, 3.0 mL solution of AOT/Brij30 in isooctane (200 mM, $\chi_B = 0.53$) containing VA or PR (concentrations given in the figure legend), 128 μL citric buffer (0.1 M, pH 4.8), and 100 μL LiP solution were transferred to a 1 cm long quartz cuvette, then 20 μL H_2O_2 aqueous solution (18 mM) was added and the resulting solution was mixed thoroughly. Periodically a 100-μL aliquot (as an enzyme solution) was taken out and its VA activity was measured using the same method as in Section 2.4 for VA oxidation. The assay conditions were as follows: 200 mM AOT/Brij30, $\chi_B = 0.53$, $\omega_0 = 23$, pH= 4.8, 10 mM VA, 10 μM H_2O_2.

2.7 Selwyn test

According to a previously reported procedure [30], the $A_{500} - t$ curves of the LiP catalyzed oxidation of PR were first recorded at several LiP concentrations (detailed experimental conditions were shown in the figure legends), and then the increment in absorbance

$(-\Delta A)$ at 500 nm at a given time (t) was plotted against (LiP concentration × time). The inactivation of LiP caused by H_2O_2 in the absence or presence of VA during the LiP catalyzed oxidation of PR could be estimated based on the degree of the superposition of curves at different LiP concentrations.

3 Results and discussions

3.1 LiP catalyzed oxidation of PR

Our previous work (unpublished results) indicated that LiP had relatively high VA activity in the mixed AOT/Brij30 reversed micelles. In such medium ([AOT] + [Brij30] = 200 mM, $\chi_B = 0.53$, $\omega_0 = 23$, pH = 4.8), LiP did catalyze the oxidative decolourization of PR by H_2O_2. As time went on, the absorbance at 500 nm (the characteristic peak of PR) decreased while the absorbance at 390 nm increased slowly. Because of the strong absorption of PR at 390 nm, we chose 500 nm to monitor the process of the LiP catalyzed oxidation of PR.

3.1.1 Effect of the concentration of PR

The initial rates of the LiP catalyzed oxidation of PR at different PR concentrations were measured. As shown in Fig. 1A, the initial rate increased with the increase of the PR concentration; when the concentration of PR was over 40 μM the curve leveled off. This indicated that LiP was saturated by PR at 40 μM or higher. From Fig. 1A an apparent $K_{m,PR}$ of ca. 20 μM was estimated. As compared with apparent Km,VA in the same medium (ca. 3 mM, see Fig. 1B), it seemed that the affinity between LiP and PR is stronger than that between LiP and VA.

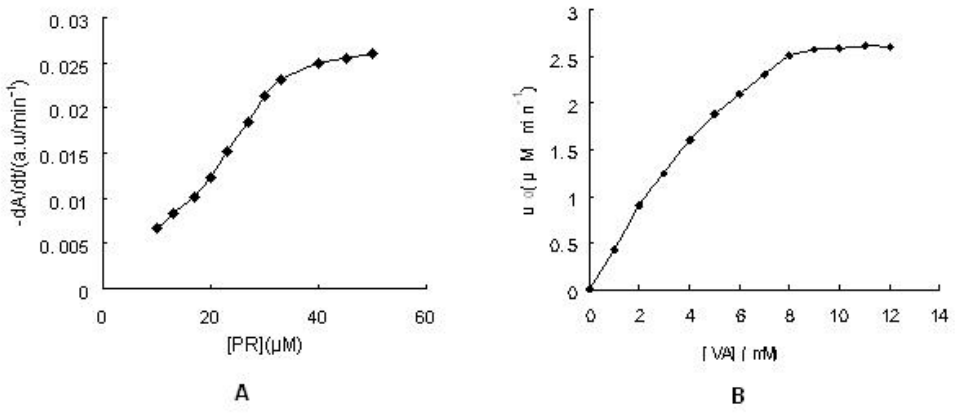

Fig. 1 Effect of the concentration of substrate on the LiP catalyzed oxidation of substrate (A, PR; B, VA). Conditions: [LiP] = 0.1 μM; [H_2O_2] = 20 μM (A), 60 μM (B).

3.1.2 Effect of H_2O_2 concentration

H_2O_2 plays dual roles in LiP catalyzed oxidations [15, 31–34] and for LiP, the inhibitory concentration of H_2O_2 varies with different substrates [15]. The effect of H_2O_2 concentration on the initial rate of the LiP catalyzed oxidation of PR was therefore studied so that an appropriate H_2O_2 concentration could be used to investigate the role of VA in the oxidation of PR. It was seen from Fig. 2 that the plot of $-dA/dt$ versus $[H_2O_2]$ was a bell-shaped curve, with an optimum H_2O_2 concentration at 15 μM. At H_2O_2 concentrations lower than the optimum value, it activated LiP; at higher concentrations, however, it inactivated LiP.

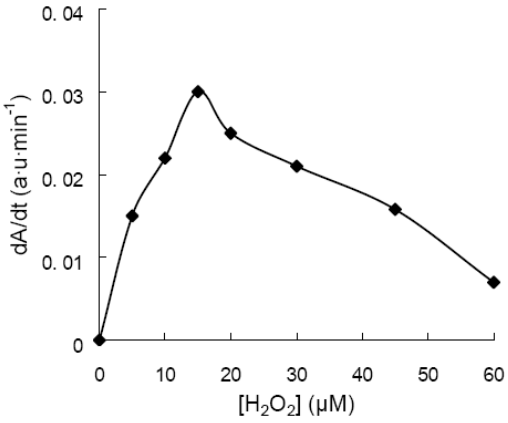

Fig. 2 Effect of the concentration of H_2O_2 on the initial rate of the LiP catalyzed oxidation of PR. Conditions: [LiP] = 0.1 μM; [PR] = 40 μM.

3.2 LiP catalyzed oxidation of PR in the presence of VA

3.2.1 Effect of VA on the LiP catalyzed oxidation of PR

In the absence of VA (other conditions same as in Fig. 3), the kinetic spectra of the LiP catalyzed oxidation of PR changed little with time (data not shown), i.e., the reaction extent of PR was very small. In the presence of VA, however, the reaction extent of PR increased (see Fig. 3), indicating that VA could stimulate the LiP catalyzed oxidation of PR.

3.2.2 Role of VA in the LiP catalyzed oxidation of PR

Based on the inhibitory concentration of H_2O_2 determined in the preceding section, three largely different H_2O_2 concentrations were selected. They were 10 μM (below the inhibitory concentration), 60 μM (above the inhibitory concentration) and 120 μM (much larger than the inhibitory concentration), respectively. At each H_2O_2 concentration, the effect of VA on the LiP catalyzed oxidation of PR was investigated (see Fig. 4). As shown in Fig. 4, the LiP catalyzed oxidation of PR was accelerated by VA at the three H_2O_2

concentrations. PR is a phenolic compound. If the conclusion of Aust *et al.* [34] (phenolic compounds could effectively reduce LiP(II) but not LiP(III) to native LiP) held true to PR, then, at H_2O_2 concentrations lower than the inhibitory concentration, VA should have had little effect on the catalytic cycle of LiP during the oxidation of PR. So we made an inference that, at low H_2O_2 concentration (10 μM), the VA induced increase of the initial rate of the LiP catalyzed oxidation of PR was caused by the indirect oxidation of PR by $VA^{+\bullet}$. The fact that the mediating effect of VA increased with increasing VA concentration (see Fig. 4) supported the above inference. With the increase of VA, more VA competed with PR for the active sites of LiP, which (although $K_{m,VA} >> K_{m,PR}$ ($K_{m,VA}$ = 3 mM, $K_{m,PR}$ = 20 μM)) resulted in more $VA^{+\bullet}$ production. As a result, more PRs were indirectly oxidized by $VA^{+\bullet}$.

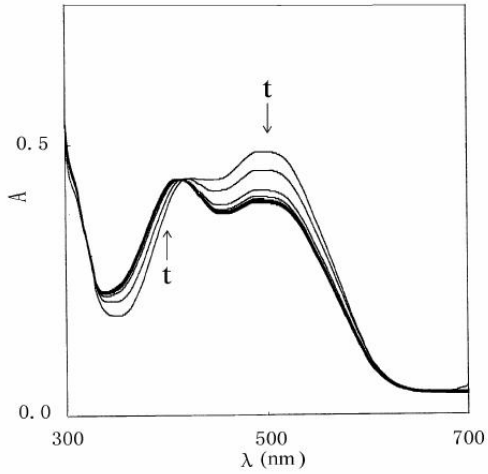

Fig. 3 Kinetic spectrum of the LiP catalyzed oxidation of PR in the presence of VA (10 mM). Conditions: [PR] = 40 μM; [LiP] = 0.1 μM; [H_2O_2] = 60 μM, the time interval for scanning was 5 min.

At a high H_2O_2 concentration (120 μM), and without VA, LiP was inactivated almost completely. In the presence of VA, however, the catalytic activity of LiP was partly restored (see Fig. 4). So it was inferred that at high H_2O_2 concentrations VA should play two roles: (i) it facilitated the conversion of LiP(III) back to native LiP [34] by reacting with LiP(III), which resulted in an active LiP to oxidize PR; (ii) using $VA^{+\bullet}$ (a strong oxidizing agent) to oxidize PR. The combined effect of VA enhanced the initial rate of the LiP catalyzed oxidation of PR [10, 14, 24].

3.2.2.1 Protection of LiP by VA In the presence of H_2O_2 and without substrates, LiP(II) is readily converted to LiP(III) [31–33], a catalytically inactive form of the enzyme, and LiP(III) can react further with H_2O_2, resulting in irreversible inactivation of the enzyme by the enzyme bleaching. In the presence of substrates, however, the reduction of LiP(II) to native LiP by the substrates (e.g. VA) helps LiP to complete its catalytic cycle; i.e., to a certain extent, substrates can protect LiP from the H_2O_2-derived inactivation.

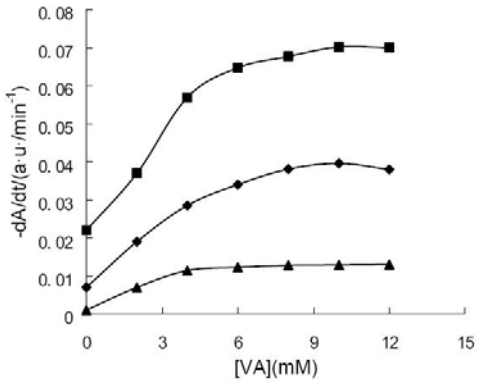

Fig. 4 Effects of the VA concentration on the initial rate of the LiP catalyzed oxidation of PR at three largely different H_2O_2 (as initiator) concentrations. Conditions: $\lambda = 500$ nm; [PR] = 40 μM; [LiP] = 0.1 μM;[H_2O_2] = 10 μM(■), 60 μM(◆), 120 μM (▲).

In order to discriminate between the two roles of VA exerted at high H_2O_2 concentrations, we studied the effect of VA on the stability of LiP in the presence of H_2O_2 and the results were shown in Fig. 5.

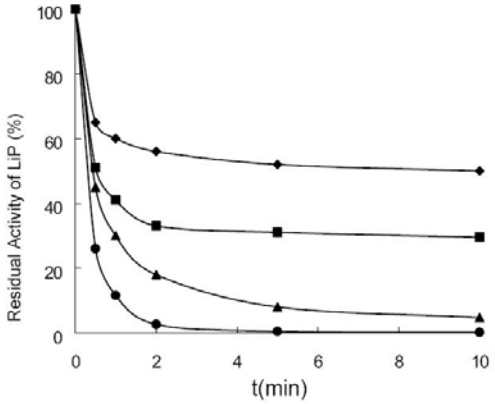

Fig. 5 Effects of VA and PR on the stability of LiP in the presence of H_2O_2. Conditions: LiP (1.0 μM) was incubated with H_2O_2 (120 μM) in the mixed reversed micelles in the absence (●) or in the presence of either VA (40 μM, ▲ ; 10 mM, ◆) or PR (40 μM, ■). Periodically, aliquots (100 μL) were removed and assayed for LiP activity as described in Methods and materials.

When LiP was incubated with 120 μM H_2O_2, almost all activity was lost within 2 min. Addition of VA could weaken the H_2O_2-derived inactivation of LiP, but the protection is not substantial at low VA concentration. Fig. 5 also showed that PR could protect LiP from the inactivation although it was a phenolic compound and, to our surprise, when the concentrations of PR and VA were both 40 μM, the effect of PR was better than that of VA. This is a novel phenomenon which has not been reported before and studies on the role of PR in protecting LiP are under way. Due to the interference of PR on the

determination of the VA activity of LiP, the protective effect of PR at high concentration could be hardly studied here. The above results indicated that in the VA mediated LiP catalyzed oxidation of PR, the role of VA as a protector was not prominent even at high H_2O_2 concentrations. This conclusion was supported by the results of Selwyn test (see Fig. 6).

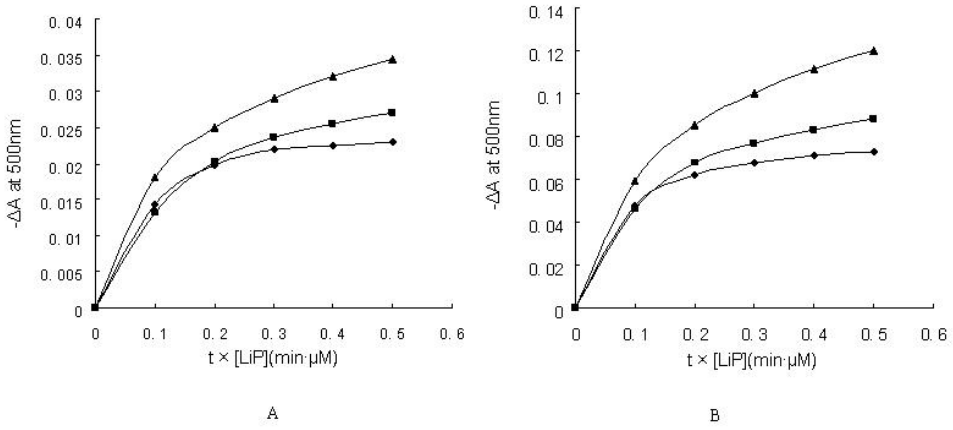

Fig. 6 Inactivation of LiP by H_2O_2 in the absence (A) or presence (B) of VA during the LiP catalyzed oxidation of PR. Conditions: First, the $A_{500} - t$ curves of the LiP catalyzed oxidation of PR (40 μM) initiated by H_2O_2 (60 μM) were recorded at several LiP concentrations ([LiP] = 0.1 μM (◆), 0.2 μM(■), 0.3 μM(▲)), then $-\Delta A$ at 500 nm was plotted against (t × [LiP]). VA = 0 mM (A), 10 mM (B).

Selwyn [30] showed that the plot of the concentration of a product formed during an enzyme catalyzed reaction versus the product of reaction time and enzyme concentration could be used to evaluate the degree of the enzyme inactivation in the course of the reaction. If curves obtained at different enzyme concentrations overlapped, then the enzyme was not inactivated during the reaction, otherwise, the enzyme was inactivated. From Figs. 6A and 6B we can see that VA could not markedly weaken the inactivation of LiP by H_2O_2 during the oxidation of PR. In fact, as time went, the $A_{310} - t$ curve of the LiP catalyzed oxidation of VA also incurvated towards the abscissa. The above results further supported our inference that in the mixed reversed micellar medium, the indirect oxidation of PR by $VA^{+\bullet}$ was the dominant factor compared to the protection of LiP by VA in the VA mediated LiP catalyzed oxidation of PR even at high H_2O_2 concentrations.

3.2.2.2 Indirect oxidation of PR by $VA^{+\bullet}$

3.2.2.2.1 Effect of the controlled release of H_2O_2 on the LiP catalyzed oxidation of PR

The inhibitory effect of phenolic compounds on LiP is mainly represented by the nonlinear variation of absorbance with time. This phenomenon is correlated with the level of H_2O_2 in the course of the reaction. The latter is related with the H_2O_2 supply strategy.

In order to discriminate between the two roles of VA exerted at high H_2O_2 concentrations and shed light on the indirect oxidation of PR by $VA^{+\bullet}$, an established enzymatic H_2O_2 supply strategy [15] was used to replace the external H_2O_2 supply strategy used in the preceding experiments. This measure ensured that the kinetic curves of the LiP catalyzed oxidation of PR were linear at a given time interval and constant total amounts of H_2O_2. In this way, the possibility of the conversion of LiP(II) to LiP(III) by H_2O_2 reduced and so did the protection of LiP by VA.

In the enzymatic H_2O_2 supply strategy, H_2O_2 came from the GOD catalyzed oxidation of glucose and the amount of H_2O_2 could be easily controlled by the amount of GOD added at a given pH. Due to the controlled release of H_2O_2, the level of H_2O_2 could be kept at a moderate level and a sustainable high LiP activity was obtained. Fig. 7 shows the kinetic spectra of the LiP catalyzed oxidation of PR in which H_2O_2 was supplied enzymatically (the amount of H_2O_2 generated in 1 h was 60 μM). The time interval used for scanning in Fig. 7A was the same as in Fig. 7B, so we could estimate the reaction rate based on the decrement in absorbance at a given wavelength. A comparison between Fig. 3 and Fig. 7A (all conditions were the same except the H_2O_2 supply strategy) indicated that the enzymatic H_2O_2 supply strategy resulted in a high and sustainable LiP activity, which made the LiP catalyzed oxidation of PR go thoroughly. This strategy did facilitate the study on the mechanism of the VA mediated LiP catalyzed oxidation of PR.

From Fig. 7A and Fig. 7B we could see, by comparison, that addition of VA greatly accelerated the LiP catalyzed oxidation of PR. Under the given conditions, the inhibitory effect of H_2O_2 on LiP was little, that is to say, the protective effect of VA on LiP was little, so the dominant effect of VA here should be the indirect oxidation of PR by $VA^{+\bullet}$.

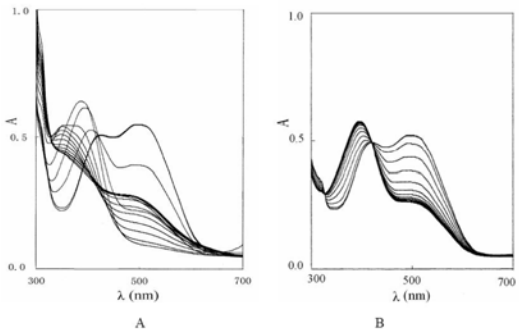

Fig. 7 Kinetic spectra of the LiP catalyzed oxidation of PR in the presence (A) or absence (B) of VA. Conditions: The LiP (0.1 μM) catalyzed oxidation of PR (40 μM) in the presence (A) or absence (B) of VA (10 mM) was initiated by H_2O_2 supplied enzymatically ([GOD] = 0.3 $\mu g \cdot mL^{-1}$, [glucose] = 0.1 mM). The time interval for scanning was 10 min.

For further elucidation of the role of VA, the stage-by-stage kinetic spectra of the system in Fig. 7A were re-recorded at different time interval at different stages of the reaction. Fig. 8a shows the kinetic spectra at the first stage of the reaction, from the initiation to the time at which the absorbance at 390 nm reached the maximum; Fig. 8b shows the spectra at the second stage, from the time at which the absorbance at 390 nm

began to decrease to the time at which the absorbance at 350 nm reached the maximum; Fig. 8c shows the spectra from the time at which the absorbance at 350 nm began to decrease onwards. As we can see from Fig. 8, there were three isosbestic points in the course of the reaction. They occurred at 418, 360 and 325 nm respectively. Also it was found that the variation of absorbance with time seemed to be linear at both sides of every isosbestic point. This phenomenon can be explained satisfactorily with the indirect oxidation of PR by $VA^{+\bullet}$. Under the conditions in Fig. 8, [VA] >> [PR] >> [LiP], so the probability of the direct oxidation of PR by LiP was much less than that of VA. Also because of the strong oxidizing power of $VA^{+\bullet}$, the indirect oxidation of PR by $VA^{+\bullet}$ took place. During the reaction, the concentration of $VA^{+\bullet}$ was relatively constant, which was about twice the concentration of LiP. As a result, the oxidative transformation of PR caused by $VA^{+\bullet}$ ran at a constant speed (attention should be paid to the Beer's law that the absorbance is directly proportional to the molar extinction coefficient as well as the concentration of light absorbing species).

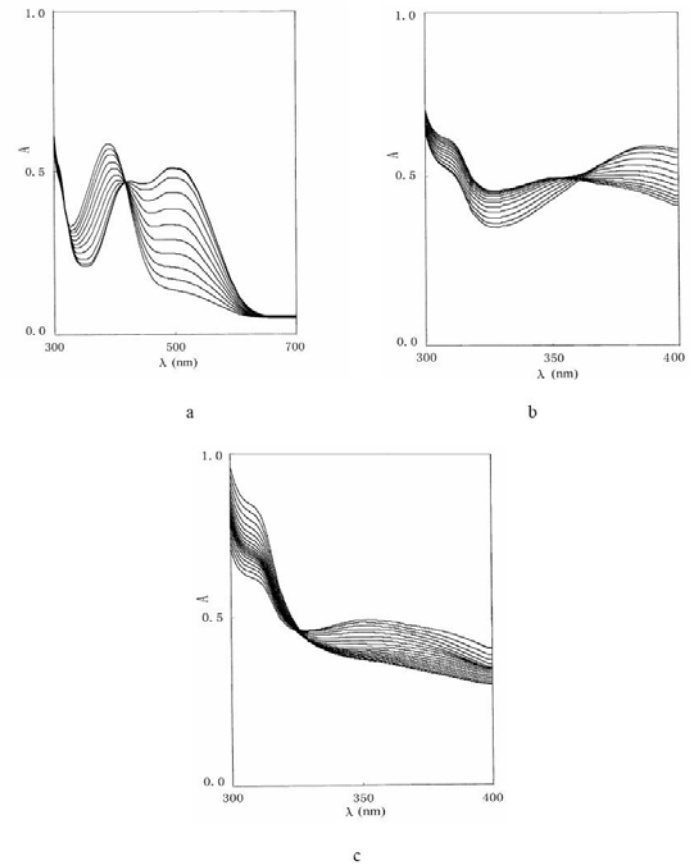

Fig. 8 Kinetic spectra of the LiP catalyzed oxidation of PR at different stage of the reaction. Conditions: [PR] = 40 μM; [LiP] = 0.1 μM; [GOD] = 0.3 $\mu g \cdot mL^{-1}$; [glucose] = 0.1 mM; [VA] = 10 mM; the time interval for scanning at the first (a), second (b) and third (c) stages of the reaction was 2.5 min (a), 1.5 min (b), and 5.0min(c), respectively.

3.2.2.2.2 Effect of PR on the LiP catalyzed oxidation of VA

For further verification of the indirect oxidation mechanism of PR, the kinetic curves of the LiP catalyzed oxidation of VA were recorded at several PR concentrations (see Fig. 9; the oxidation product of VA, veratraldehyde, has maximum absorption at 310 nm). Theoretically, the reduction of $VA^{+\bullet}$ by PR should retard the formation of veratraldehyde and, therefore, there should be a lag period in the $\Delta A_{310} - t$ curve and, moreover, the duration, if other conditions were fixed, should be directly proportional to the PR concentration. Fig. 9 indicated that in the presence of PR, there existed a lag period in the kinetic curves, especially at high PR concentrations. Although these kinetic curves were no longer linear, they were similar in shape; i.e., there were two periods in which the absorbance increased rapidly. To investigate the cause that resulted in the rapid increase of absorbance in both periods, we recorded several $A - t$ curves at several special wavelengths ([PR] was kept at 40 μM). Curve 1 (λ = 310 nm) in Fig. 10 showed that there were two periods during which the absorbance increased rapidly. The first occurred at about 14 min after the initiation (the left triangle in curve 1) and lasted about 12 min; the second occurred at about 50 min (the right triangle in curve 1), and from then on, the absorbance increased with time. As shown in Fig. 10, at 14 min curve 3 (λ = 390 nm) began to decline while the peak of curve 2 (λ = 350 nm) began to shape up, so we speculated that, from this time on, the increase in absorbance at 310 nm was caused by an intermediate of PR with characteristic absorption peak at 350 nm, not by veratraldehyde; at 50 min, the PR oxidation was almost completely finished (see curves 2, 3 and 4), and from then on, the increase in absorbance at 310 nm should be caused by veratraldehyde with characteristic absorption peak at 310 nm. These results suggested that PR indeed inhibited the oxidation of VA and delayed the formation of veratraldehyde. The time interval just before the formation of veratraldehyde was here called the inhibitory time. Fig. 9 showed that the inhibitory time did increase with the increase of PR concentration, which made the accurate determination of the VA activity of LiP in the presence of high concentration of PR difficult.

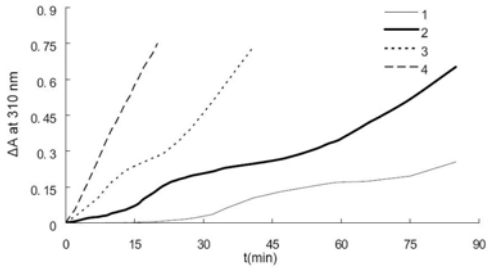

Fig. 9 Kinetic curves of the LiP catalyzed oxidation of VA at several PR concentrations. Conditions: The LiP (0.2 μM) catalyzed oxidation of VA (10 mM) in the presence of PR ([PR] = 55 (1), 40 (2), 20 (3), 0 (4) μM) was initiated by H_2O_2 supplied enzymatically ([GOD] = 0.3 $\mu g \cdot mL^{-1}$, [glucose] = 0.1 mM) and monitored spectrophotometrically at 310 nm.

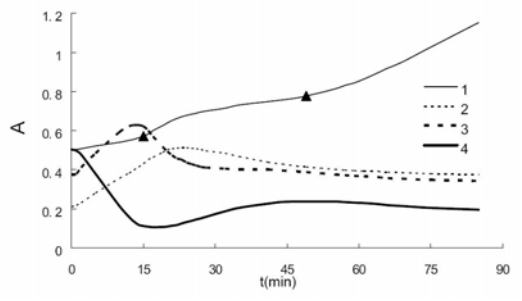

Fig. 10 A-t curves of the LiP catalyzed oxidation of VA in the presence of PR (40 μM). Conditions: The reaction conditions were the same as in Fig. 9. [LiP] = 0.2 μM; [GOD] = 0.3 $\mu g \cdot mL^{-1}$; [glucose] = 0.1 mM; [VA] = 10 mM; λ=310 nm (1), 350 nm (2), 390 nm (3), 500 nm (4).

4 Conclusions

Based on the above study, the following conclusions were drawn:

(1) VA could accelerate the LiP catalyzed oxidation of PR, especially at low H_2O_2 concentrations.
(2) At the same protector concentration, VA was less effective than PR in protecting LiP from the H_2O_2 derived inactivation.
(3) The enhancement of the PR oxidation caused by VA was mainly due to the indirect oxidation of PR by $VA^{+\bullet}$.

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