Hematin-Catalyzed Polymerization of Phenol Compounds

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ABSTRACT: The oxidative dehydrogenation of phenol compounds, as well as polymer formation from these monomers, was studied by UV−vis and Mössbauer spectroscopy using a novel biological catalyst hematin. The mechanism of the polymerization reaction was also followed in various pH environments. Phenol radicals were formed by a two-step electron transfer reaction catalyzed by hematin in the presence of peroxide, and polymer was formed from phenol radicals by a noncatalytic reaction. This mechanism partially explains the analogous catalytic polymerization activity of horseradish peroxidase.

Introduction

A number of investigations have been conducted toward the peroxidase-catalyzed polymerization of phenols, aromatic anilines, and their derivatives in various media.[1−12] This polymerization method provides an environmental friendly, economic, and safe methodology to prepare polymers with different functional groups. Phenolic monomers, with varying substitutents on the aromatic ring, undergo oxidative dehydrogenation reactions catalyzed by an enzyme, thereby enabling the modification of polyphenol properties. With the additional substitution of some elements such as bromine and phosphorus on the aromatic ring, polyphenols synthesized by this method have potential fire-retardancy, as applied in many phenol−formaldehyde resins.[13]

The peroxidases are widely distributed in nature, and horseradish peroxidase (HRP) is commercially important. HRP is a group of isoenzymes with molecular masses in the range 38−42 kDas, with an isoelectric point of 7.2 and pH stability between 4.5 and 12.[14] One of the major isoenzymes of horseradish peroxidase, HRP-C, has special affinity for phenol compounds, and these monomers preferentially bind with the active site of the enzyme before the oxidative dehydrogenation. In the presence of organic hydroperoxides or hydrogen peroxide the enzyme is oxidized to compound **I** (Scheme 1) which in turn oxidizes phenols and their derivatives according to the following equation:

$$n\mathrm{AH} + (n/2)\mathrm{H_2O_2} \rightarrow \bullet\mathrm{A}-(\mathrm{A}-\mathrm{A})_{n-1} + n\mathrm{H_2O}$$

The mechanism and catalytic cycle of horseradish peroxidase, a classic example of an enzyme used to catalyze phenol compounds, are fairly well established.[1−12,14] The enzyme utilizes a heme−iron cofactor to interact with the peroxide, yielding an oxidized heme−iron complex **I** that subsequently react with the substrate in a one-electron-transfer reaction to produce a substrate radical and a further heme−iron complex **II** (Scheme 1). The changes of oxidation state of iron in the heme invoke an alternative for HRP.

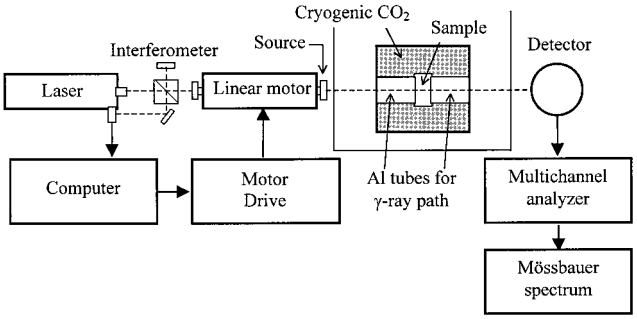
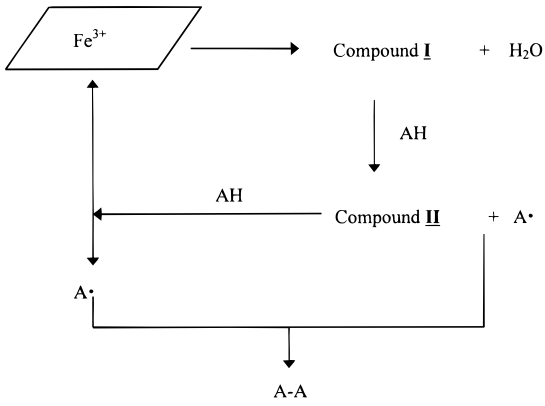


Figure 1. Schematic diagram of the Mössbauer spectrometer.

Scheme 1. Polymerization Mechanism Catalyzed by HRP[12]



Heme is a ferriprotoporphyrin with no charge, and hematin is hydroxyferriprotoporphyrin with the structures depicted in Figure 2. The free heme is unstable and is rapidly oxidized to hematin. In this work, we used hematin as a promising alternative to replace HRP for the polymerization of phenol derivatives. Hematin is a small molecule with a much lower cost than that of HRP, and hematin-catalyzed polymerization of phenol derivatives would provide similar advantages as HRP while decreasing the catalyst cost.

In this work, the catalytic mechanism of hematin was investigated by Mössbauer spectroscopy coupled with UV−vis. The Mössbauer effect (recoil-free energy nuclear

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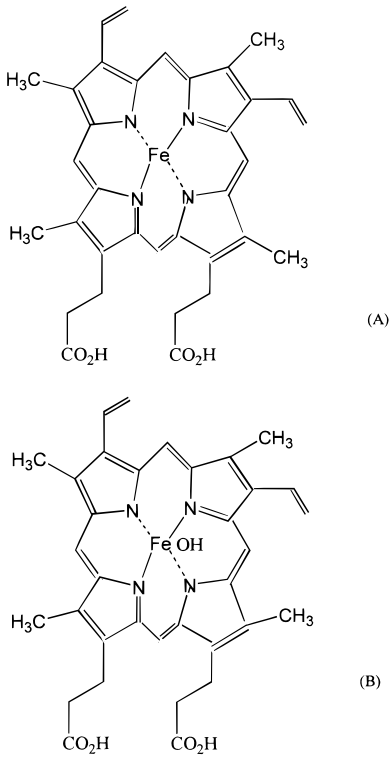


Figure 2. Structures of (A) heme and (B) hematin.

γ-ray resonance) has been used to study many interesting problems in physics and chemistry.[15−17] It appears that only the elements iron and iodine offer reasonable possibilities for Mössbauer experiments of a biological nature. Fe^{57} has become the most widely used Mössbauer isotope in physics and chemistry, and it is fortunate that iron is contained in hemoglobin, myoglobin, cytochrome, peroxidases of plants, catalases of erythrocytes, and other molecules which have interesting biological functions.[18] This technique enables a nondestructive measurement on an atomic scale and to single out and the study of only the iron atoms in the sample.

Experimental Section

Materials. Hematin and horseradish peroxidase (HRP) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethylphenol (EP) and hydrogen peroxide (H_2O_2) were obtained from Aldrich Chemical Co. (Milwaukee, WI). They were used as received. Buffers of sodium succinate (0.1 M, pH 4.0), sodium phosphate (0.1 M, pH = 7.0), and sodium carbonate (0.1 M, pH = 11.0) were used in the kinetic and polymerization studies.

Mössbauer Setup. The basic components of apparatus are shown schematically in Figure 1. A computer-controlled linear motor drove a ^{57}Co source in both forward and reverse directions at constant acceleration. The instantaneous velocity was measured by the Michelson interferometer using a laser and calibrated using pure iron-foil at room temperature. In all experiments, the absorber was stationary. The detector was a krypton proportional counter biased at 1800 V. Two stages of amplification and discrimination of signals were used, and the pulses produced by the 14.4 keV γ-ray were stored in a 512-multichannel analyzer. In these experiments, the velocity limits were set at 4.00 mm/s.

Characterization. FTIR spectra were obtained on a Nicolet 60SX spectrometer. ^1H NMR spectra were performed on a Brüker AF-400 spectrometer. UV−vis analysis was conducted on a Beckman DU7500 spectrophotometer. The molecular masses and molecular mass distributions of the polymers were determined using gel permeation chromatography (GPC). The GPC analysis was conducted in 1 wt % LiBr/DMF with a Waters LC Module 1. Narrow molecular mass styrene standards ranging from 400 to 9300 Da were used for calibration.

Polymerization of EP Catalyzed by Hematin. (a) Hematin Activity. Varying amounts of hematin (6.3, 4.5, 3.2, and 1.6 mg for 2, 1.5, 1, and 0.5 mM, respectively) were dissolved in 1.5 mL of DMF, and this was facilitated by sonication. The activity of hematin was measured by adding H_2O_2 to the monomer solution (ethylphenol, 1 mmol, 0.12 g) prepared in 3.5 mL of a buffer solution. The reaction mixture was stirred for 1 h at room temperature. Hydrogen peroxide, 0.15 mL, was added dropwise three times, with 15 min intervals to initiate the reaction. The reaction was maintained for 24 h. The precipitation of dark products was observed, indicating formation of polymer. The products were collected by centrifugation and followed by washing with sodium carbonate buffer and then water. The yield of poly(*p*-ethylphenol) (PEP) and molecular masses are listed in Table 1. The reaction was carried out at different pH values (4.0, 5.0, 7.0, 8.0 11.0) solutions to study the effect of pH on PEP synthesis.

(b) Polymerization Rate of EP. The reaction rate of hematin-catalyzed EP polymerization was studied by terminating the reaction at various time periods. The polymerization procedure was similar to that above with 1 mM hematin.

Mechanism Study by Mössbauer and UV−Vis Spectroscopy. A. Hematin in Buffer Solution for Mössbauer Effect. The main difficulty of applying this technique to biophysical studies concerns the extreme dilution of presently available Mössbauer isotopes in most biological compounds. However, this difficulty is an experimental problem which can usually be solved (if necessary with the use of enriched isotopes) and should not prevent the Mössbauer effect from becoming a new and interesting tool for biological research. In the work, we used a large amount of hematin to obtain Mössbauer spectra.

A 400 mg sample of crystalline hematin was placed in a plastic tube, and the spectra were obtained at room temperature after 24 h. To acquire spectra of a solution sample, 180−200 mg of hematin was dissolved in 5 mL of sodium phosphate buffer solution (pH = 11.0) in the plastic tube, which was then placed in dry ice to freeze the sample. Subsequently the frozen sample was mounted into the Mössbauer spectrometer and spectra were collected after 48 h. Later 0.2 mL of H_2O_2 was added into the melted hematin solution. Effervescence was observed, indicating the interaction of H_2O_2 with hematin. The sample was frozen again and mounted into the Mössbauer spectrometer to acquire spectra.

B. UV−Vis Analysis of Hematin Reaction with H_2O_2. Hematin, 3 mg, was dissolved in 3 mL of DMF. In a 1 mL cuvette, 50 μL of hematin solution was diluted by 1 mL of a buffer solution to give a concentration of 50 ppm. A stoichiometric amount of H_2O_2 (4.5 μL) was injected into the hematin solution in a cuvette. Immediately the reaction was monitored and recorded by UV−vis spectroscopy at room temperature during varying time periods.

C. UV−Vis Analysis of Hematin-Catalyzed EP Polymerization. This procedure was similar to part B, except hematin (50 μL) was diluted in a cuvette with 1 mL of 0.1 mM EP solution. The ethylphenol solution was prepared by mixing 63 mg of EP in 2 mL of DMF with 3 mL of buffer solution).

D. UV−Vis Analysis of HRP Reacted with H_2O_2 at pH = 7.0. To compare the reaction between HRP and H_2O_2, a

Table 1. Effect of Hematin Concentration on Polymerization (pH = 11.0)

$[hematin]_0$ (mM)	2	1.8	1.5	1.2	1	0.8	0.5
EP yield (%)	50	45	50	70	72	60	40
M_w/M_n	2240/1480	2210/1480	2370/1500	1700/1220	1680/1150	1600/1100	1610/1150
DP	1.50	1.50	1.58	1.39	1.46	1.45	1.40

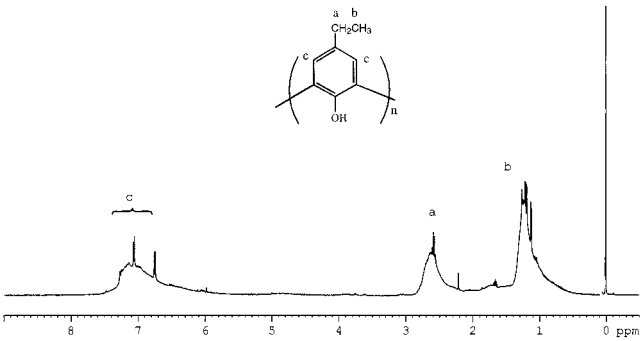


Figure 3. ^{1}H NMR spectrum of PEP obtained by hematin catalysis.

Table 2. Effect of pH on Polymerization of EP

pH	4.0	5.0	7.0	8.0	11.0
EP yield (%)			50	61	72
M_w/M_n			1870/1060	1710/1130	1680/1150
DP			1.76	1.51	1.46

UV−vis monitoring procedure was performed for HRP similar to that described in part A. Since HRP is a very large molecule and very active toward H_2O_2, 50 μL of H_2O_2 (30%) was diluted by 2.95 mL of H_2O. Subsequently, diluted H_2O_2 (5 μL) was used to react with 100 μL of HRP (pH 7.0 buffer with HRP 1 mg/mL) solution.

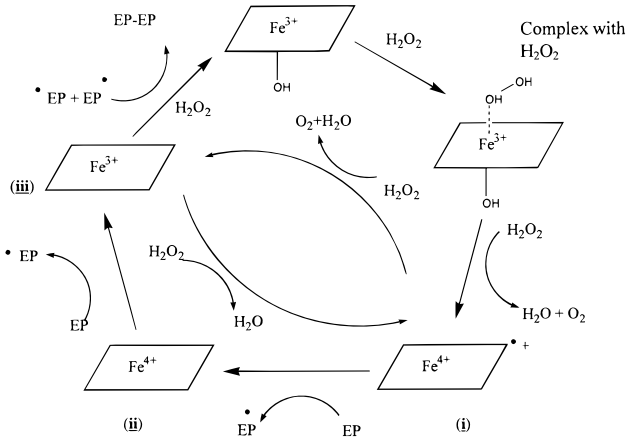
E. UV−Vis Analysis of HRP-Catalyzed EP Polymerization. A 3 mg sample of HRP was dissolved in a buffer solution (pH = 7.0). EP (63 mg) was dissolved in 2 mL of DMF and 3 mL of buffer solution to make a 0.1 mM EP solution. H_2O_2 (30%) was diluted as described in part D above. Then, 100 μL of HRP buffer solution was pipetted into a 1 mL cuvette and diluted by 1 mL of EP solution. Afterward, 5 μL of diluted H_2O_2 was added into the cuvette to initialize the polymerization. Immediately the reaction was monitored by UV−vis spectroscopy.

Results and Discussion

Polymerization Ethylphenol Catalyzed by Hematin. The structure of poly(*p*-ethylphenol) (PEP) from hematin-catalyzed polymerization was characterized by FTIR and NMR. In the ^{1}H NMR spectrum (Figure 3), major peaks appeared at 1.22 ppm (3H from CH_3), 2.60 ppm (2H from CH_2) and 6.74−7.19 ppm (aromatic protons). The FTIR spectrum of PEP showed a large −OH stretch at 3340 cm^{-1}, indicating the intact OH group on PEP. The strong stretch at 1210 cm^{-1} is ascribed to the C−OH group, and the peaks at 1580, 1500, and 1460 cm^{-1} are assigned to the aromatic C=C stretch.

Hematin catalytic activity on the polymerization of EP at pH = 11.0 is listed in Table 1. It shows that the polymerization yield increased with the hematin concentration, then reached a maximum yield at 1 mM. Further increase lowered the yield. This was probably caused by the increased decomposition of hydrogen peroxide when encountering more hematin. The molecular masses were comparable to that of PEP obtained from HRP (pH = 7.0−7.5) catalysis with M_w/M_n = 2200−1600/1600−1000. The analysis of the filtrate by IR and GPC showed very little monomers or low oligomers, e.g., dimer, trimer.

Scheme 2. Proposed Mechanism of Hematin-Catalyzed Polymerization



The pH effect on the polymerization of EP is shown in Table 2. Polymerization yields increased with pH values. In addition, the dispersity index of polymer obtained in high pH value environments more likely approaches unity, indicating that basic environments favor the formation of uniform polymers. The low activity of hematin at low pH is due to its limited solubility, which restricted its activity. For further study, pH = 11.0 was used because hematin had a higher solubility and activity.

HRP-catalyzed polymerization of EP proceeds very promptly. The reaction is completed instantly.[3−4] The reaction rate of hematin-catalyzed EP polymerization was slower than that of HRP, but still the reaction are almost complete within 1 h, probably due to the rapid coupling reaction between EP• moieties. The percent yield keeps increasing with time until after 8 h and reaches the flat value of 70−72%; it is possible that polymers are developed and precipitated with time increasing. The polymer dispersity index increases with time from nearly unity (1.07) to 1.4 after 24 h; this probably results from the interexchange of polymer chains. The reaction yields at different time periods are shown in Table 3.

Mechanism Study of Hematin Catalysis. As discussed previously, intermediate compounds **I** and **II** form during HRP-catalyzed polymerization, and their exact nature is difficult to describe unambiguously by conventional valence bond representations. This may have been responsible for some confusion about their identities.[12] Therefore, here we have used the ionic state (Fe^{3+}) to represent the hematin iron state.

Hematin is a hydroxyferriprotoporphyrin with Fe^{3+} high spin state.[18] As shown in Scheme 2, the hydroxyl OH group in the hematin molecule is cleaved and gas (O_2) is released upon the addition of H_2O_2. Meanwhile intermediate states are established. This was confirmed by Mössbauer spectroscopy. Parts a and b of Figure 4 show the spectra of crystalline hematin and solution, respectively. Both asymmetrical two-line spectra could arise from quadrupole splitting (peak separation) with different δ's (peak shifts as in NMR). The lack of symmetry of the spectra indicates that it is not a simple

Table 3. Polymerization Rate of EP (pH = 11.0)

time (h)	1/6	1/2	1	5	8	24
yield (%)	42	58	60	70	72	72
M_w/M_n	1240/1160	1240/1200	1390/1210	1390/1210	1570/1190	1680/1150
DP	1.07	1.03	1.15	1.15	1.32	1.46

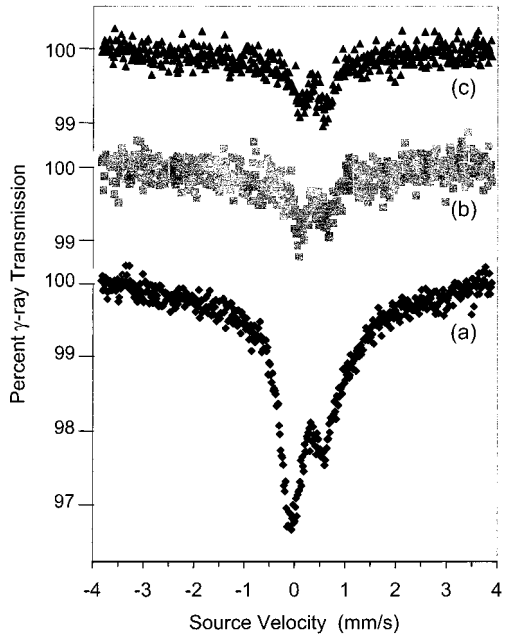


Figure 4. Mössbauer spectra of (a) cystalline hematin, (b) hematin solution, and (c) hematin solution upon the addition of H_2O_2.

doublet but there are two environments. This should be applicable because hematin is not spin paired. Heme can be viewed as having the iron atom in the porphyrin ring located in an axially symmetric configuration (probably a 4-fold rotational axis) to a first approximation, at least with respect to its nearest neighbors. Hematin, however, is a hydroxyferriprotoporphyrin and has a hydroxyl group adjacent to the iron atom. Thus, it gave asymmetrical lines.

A polymerization mechanism catalyzed by hematin is proposed in Scheme 2. Upon the addition of H_2O_2, hematin initially forms a complex. Subsequently, the hydroxyl group is cleaved, and O_2 is produced due to the cleavage of the peroxy O−O bond. Fe^{3+} loses electrons to H_2O_2, and meanwhile, hematin is transformed to the intermediate (**i**). The latter immediately withdraws an electron from EP to form **ii**, and from **ii** to form **iii**, subsequently; Fe^{4+} states are proposed.[19] **i** and **ii** are extremely active and unstable, and they withdraw electrons from the substrate EP, and eventually form the intermediate (**iii**) Fe^{3+}, which is later converted back to hematin. This was confirmed by the Mössbauer spectrum (Figure 4c). The Fe^{57} signal peaks is attributed to intermediate **iii** resulting from **i** or **ii**. The quadrupole splitting is narrow and the peak intensities are reversed, compared to Figure 4b. These changes indicate the formation of intermediate **iii**. The isomeric shift of hematin (0.5 s/mm) falls in the region of other high-spin Fe^{3+} compounds.[18] Therefore, in the presence of the substrate EP, electrons from EP are drawn to add to **i**, resulting in **ii**; **ii** subsequently withdraws electrons from EP to give **iii**. EP$^\bullet$ is formed and intermediate **iii** is recycled to the original hematin. In the absence of a substrate EP, intermediate **i** would transform into **iii**, releasing O_2 gas by withdrawing electrons from H_2O_2. This has been verified by Mössbauer spectroscopy (Figure 4c is assigned to the intermediate state **iii**).

Thus, the mechanism proposes the formation of intermediate state **ii** resulting from hematin and it is similar to compound **I**, which is best described as an iron (Fe^{3+}) porphyrin π-cation radical, in the mechanism of HRP-catalyzed polymerization (Scheme 1).[12] This mechanism was further confirmed by UV analysis. As shown in Figure 5 (pH = 7.0), hematin has an absorption at 387 nm. Upon addition of H_2O_2, the intensity of the peaks decreased with time, indicating that a reaction occurred between hematin and H_2O_2 .

In the presence of substrate (EP), hematin absorbance at 387 nm decreased initially (Figure 6a), then increased again (Figure 6b). Meanwhile the absorption range was broadened (Figure 6b); it was also observed that the solution in the cuvette gradually transformed from clear, transparent to opaque, and precipitation was observed, indicative of polymer formation. This can be rationalized as follows: while hematin absorbance at 387 nm decreased upon the addition of H_2O_2, intermediate **i** was produced, and it immediately withdrew electrons from monomer EP to form **ii** and EP$^\bullet$. Subsequently **ii** withdrew electrons from EP again to generate new EP$^\bullet$. The latter formed the oligomer PEP while encountering another EP$^\bullet$ as shown in Scheme 2. We concluded that intermediate **ii** was responsible for the information on PEP. When intermediate **ii** reached a high concentration, more PEP with high molecular masses were precipitated. Because of the conjugation effects, UV spectra show the broadened range from 350 to 525 nm, which is consistent with the observations.

To compare with HRP, and further illustrate the proposed mechanism, the reaction (pH = 7.0) between HRP and H_2O_2 was monitored by UV−vis spectroscopy. Figure 7 shows the red shift of peak from 400 to 420 nm and the decreasing of peak intensity. The red shift corresponds to compound **I** or **II** in Scheme 1 and

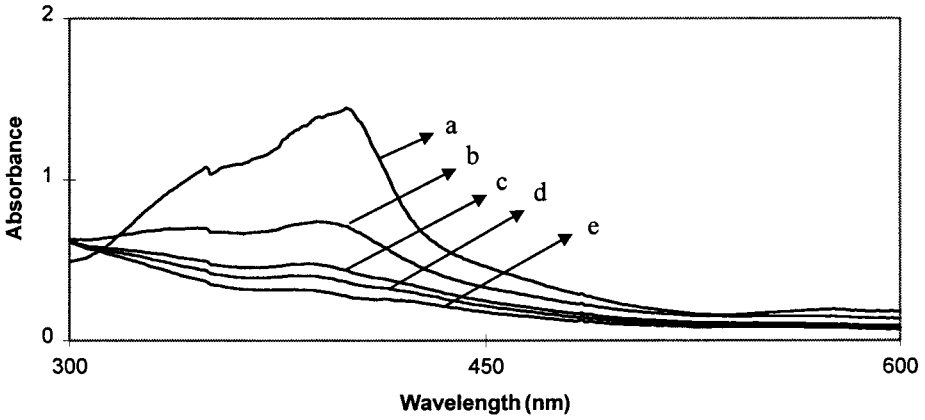


Figure 5. UV spectra of hematin buffer solution (pH = 7.0) reacted with H_2O_2: (a) 0, (b) 0.3, (c) 1, (d) 2, and (e) 4 min.

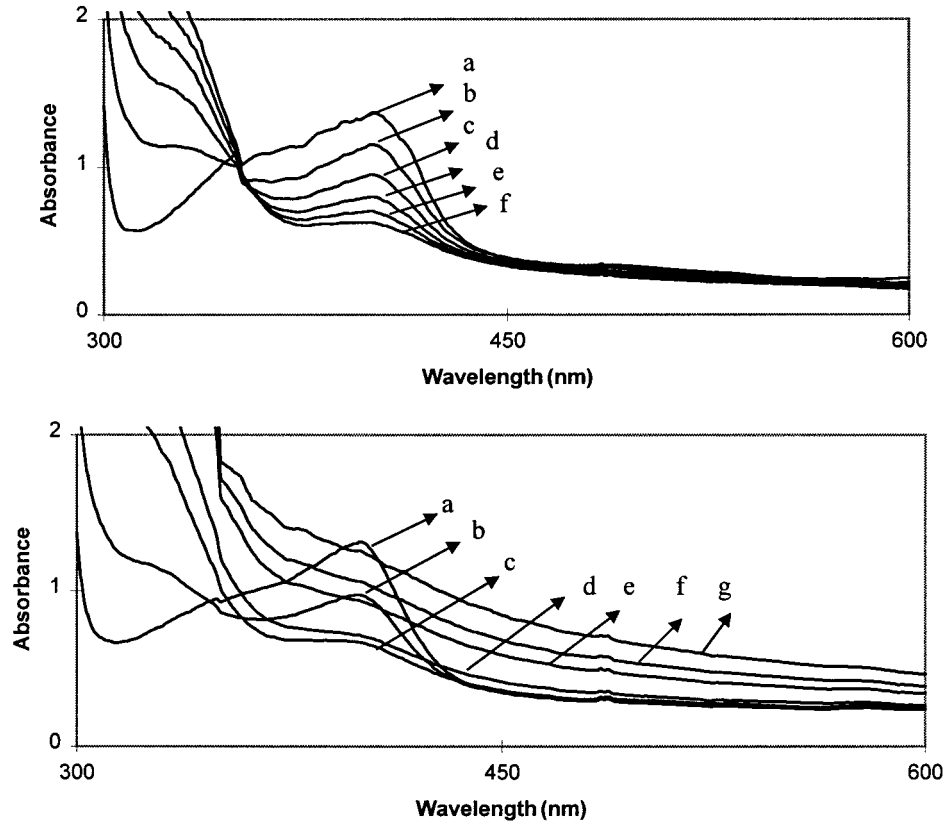


Figure 6. (a) UV spectra of EP polymerization in hematin buffer solution (pH = 7.0) initialized by H_2O_2 at an early stage: (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, and (f) 5 min. (b) UV spectra of EP polymerization in hematin buffer solution (pH = 7.0) initialized by H_2O_2 at a late stage: (a) 0, (b) 5, (c) 12, (d) 25, (e) 35, and (f) 45 min.

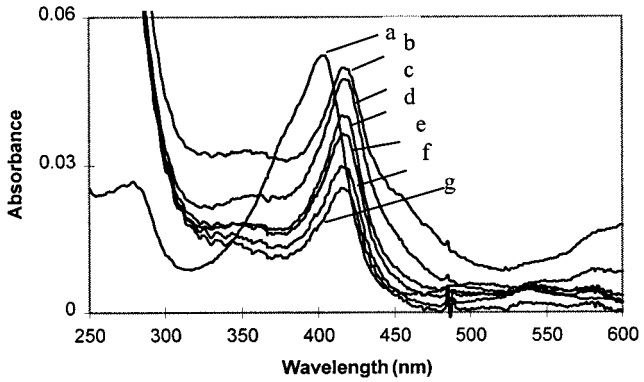


Figure 7. UV spectra of HRP reaction with H_2O_2 (pH = 7.0) after (a) 0, (b) 1, (c) 2, (d) 3, (e) 13, (f) 17, and (g) 21 min.

indicates that HRP was oxidized by H_2O_2 to the oxidant state. The decreasing peak intensity illustrated that HRP was consumed by H_2O_2 combined with the following: the color of HRP buffer solution changed from brown-orange to clear and the HRP−H_2O_2 mixture could not catalyze polymerization. In the presence of EP, compound **I** withdraws a single electron from EP and is converted to compound **II**, and PEP was produced promptly, as shown by UV−vis spectroscopy in Figure 8. This mechanism has been well studied.[1−12] From a comparison of both biologically active enzymes, we conclude that both catalysts have similar mechanisms.

Conclusion

Hematin-catalyzed polymerization shows that hematin is a promising and alternative catalyst to horserad-

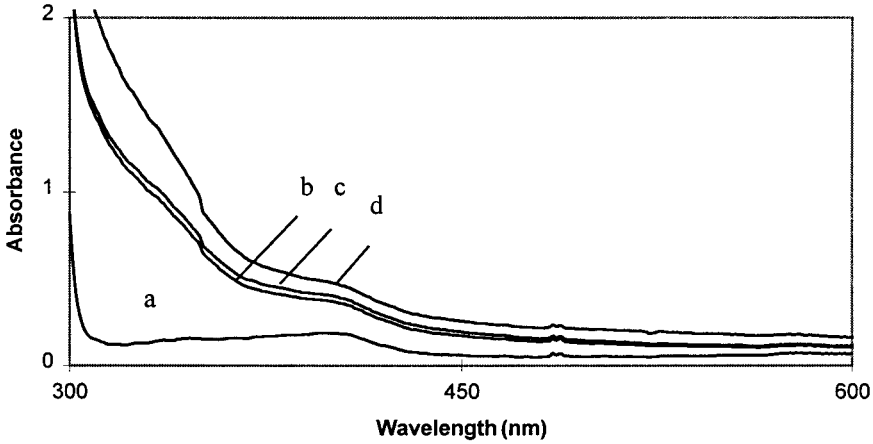


Figure 8. UV spectra of EP polymerization in HRP buffer solution (pH = 7.0) initialized by H_2O_2: (a) 0, (b) 5, (c) 10, and (d) 15 s.

ish peroxidase. Hematin is suitable in pH = 11.0 buffer solution with good activity and molecular masses. The mechanism study indicated that intermediates were produced during polymerization which are responsible for polymer formation.

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