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Horseradish peroxidase catalyzed free radical cannot free move in reaction solution

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Abstract

Mechanism of Horseradish Peroxidase -catalyzed phenol compound oxidizing reaction is a radical polymerization. Many polymer preparation are also carry on through the radical polymerization mechanism. We deduce if free radical produced by peroxidase-catalyzed phenol polymerization could apply on polymer preparation? Could the phenol–oxygen free radical leave off the peroxidase and catalyze other compounds polymerization? The free radical in phenol oxidation process was investigated in homogeneous reaction and in immobilized HRP catalyzed reaction. The results showed the free radical produced by peroxidase only move on the surface of enzyme, can't free move in solution in both experiment. Evidence showed the phenol polymerization is enzyme reaction process, different from general chemistry free radical chain reaction.
Keywords: Horseradish Peroxidase, free radical polymerization, mechanism

Introduction

Peroxidase widely exists in the higher plant, richest in the fig sap and the horse radish. horseradish peroxidase (HRP, E.C.1.11.1.7) separated from the horse radish is the most commonly used peroxidase. HRP, molecular weight 40000, isoelectric point 7.2, most suitable pH (in aqueous phase) 7.0, and take the hard heme as the prosthetic groups. In the catalyzed process, HRP responded with the hydrogen peroxide specificity, phenols compound and aromatic amines as hydrogen donor, the enzyme-oxygen free radical formed after providing H, then rearrangementing and coupling to form Oligomers(Gajhede *et al* 1997; Hernandez-Ruiz *et al* 2001; Keilin *et al* 1951; Meunier *et al* 1998; Nakajima *et al*1987).

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Horseradish peroxidase catalysis's phenol compound oxidizing reaction is one kind of radical polymerization response(Araiso *et al* 1976; Gunilla *et al* 2005; Harris *et al* 1993; Jones 2001; Keilin *et al* 1955). Many polymer preparation are also carry on through the radical polymerization reaction. So we once attempted copolymerization of phenol and styrene, acrylic amide, methyl methacrylate to synthesis functional polymer by peroxidase catalyzed, initiated by H_2O_2. Although experiments repeatedly, the free radical produced by peroxidase catalysis hydrogen peroxide cannot initiate styrene, acrylamide, methyl methacrylate polymerization, and reaction that peroxidase catalyzes the phenol compound (p-Phenylphenol, p-Methyiphenol) with styrene, acrylamide, methyl methylacrylate copolymerization also cannot carry on. So hypothesized the free radical can't free move produced by peroxidase catalysis hydrogen peroxide, two experiments were designed to prove the idea.

Experimental

Material and Characterization

Horseradish peroxidase (HRP E.C.1.11.1.7), 225 units/mg (25 ^{o}C, guaiacol, H_2O_2) was obtained from Roche (Mannheim, Germany). ESR spectra were measured on Bruker ESR 200D, 9.8 GHz, 100 KHz, ESR Spectra of free radical caught by DMPO (5.5-dimethyl-1-proline N-Oxied).

Preparation of HRP immobilization

After washing with NaOH, the self-made agar-agar bead were resuspended in 12 ml acetone, add Cyanuric chloride dissolved with dioxane and acetone. The reaction mixture was stirred for 3 min. Then, add 20% acetic acid (v/v) to stop the reaction. Wash with acetone and water separately. Add HRP to the activated agar-agar bead for reaction 3 hour. Immobilized HRP was obtained after wash the carrier with water to eliminate the residuary enzyme. The activity of immobilized HRP is 67.8U/mg, the coupling rate is 86.87%, the coupling quantity is the 7.819mgPr/g agar-agar, enzyme vigor recycling is 24%, enzyme recovery is 24%.(Akkara *et al* 1991; Kumar *et al* 2005; Livage *et al* 2001; Wilson *et al* 1986)

HRP activity

A mixture of 0.23ml phosphoric acid buffer (pH6.0,10mmol/L), 0.1 1ml hydrogen peroxide solution(0.147mol.l^{-1}), 0.32ml trihydroxybe nzene solution (5%,v/v), 2.1ml distilled water were stirred in quartz cell at 20 . Air or the distilled water as the blank control, add 0.1ml HRP, blending, measure A_{420}nm increasing rate (Bergmeyer 1975).

Free Radical Detection in Homogeneous Reaction

Add HRP in dialysis bag (blocking molecular weigh 15000D).Put dialysis bag in a 250 ml beaker. Then pour substrate (p-Phenylphenol, sigma) and H_2O_2 into solution outside dialysis bag dissovled in 70 % Dioxane/Water (v/v). Detect ESR spectra of free radical in or out dialysis bag (Fig 1.1).

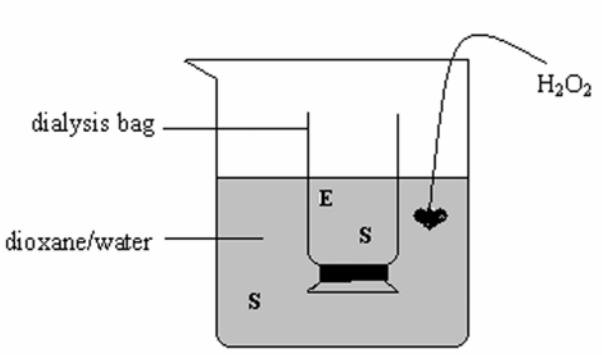

Fig 1.1 Scheme of enzymatic polymerization of p-phenylphenol in a dialysis bag. The reaction medium is the mixture of 70% dioxane and 30% water mixture containing 0.05 mol. l^{-1} p-phenylphenol. The reaction was initiated by the addition of 1 μ l , 0.1 mol.l^{-1} H_2O_2 to the outside of the dialysis bag.

Free Radical Detection in Immobilized HRP catalyzed Reaction

In reaction mixtures, add certain solubility substrate (p-Phenylphenol, sigma) and the immobilized HRP in 70%Dioxane/Water (v/v), H_2O_2 added later. Put the immobilized HRP in the capillary vessel base, detect the ESR spectra of free radical when the capillary vessel to be in or to be out from the resonant cavity (Fig 2.1).

Results and Discussion

Free Radical Detection in Homogeneous Reaction

In order to examine free radical which produced by peroxidase-catalyzed hydrogen peroxide can free move or not in the reaction, we add HRP to the dialysis bag (blocking molecular weight 15000D). Put substrate and H_2O_2 outside the dialysis bag. HRP(MW40000D) can't permeate the bag, and substrate p-Phenylphenol (MW170D) can free move in or out dialysis bag. Precipitation was observed in the bag to approve the substrate move into the bag interior. Reacting 3 hour later, solution outside dialysis bag was token out to detect. No polymer has been discovered by HPLC analysis. At the same time, we also tested by the paramagnetic, discovered that the dialysis bag interior solution had the free radical signal, but the exterior solution did not have. (Fig 1.2)

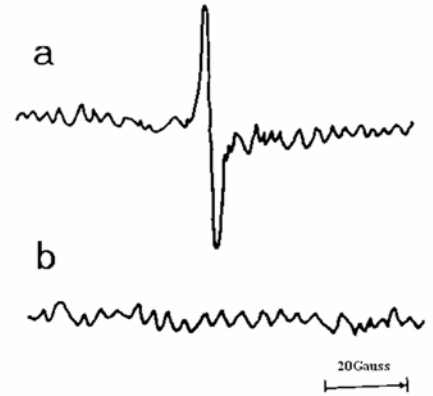

Fig 1.2 The ESR spectrum of the solution: (a) inside and (b) outside the dialysis bag.

By HPLC and ESR, we thought that the free radical polymerization is limited in dialysis bag interior, does not enter outside in the dialysis bag's solution in the reaction process. So deduce that life of phenol oxygen free radical and phenols' free radical are short, phenol oxygen free radical's formation as well as later rearrangement, coupling, polymerization carry on nearby the

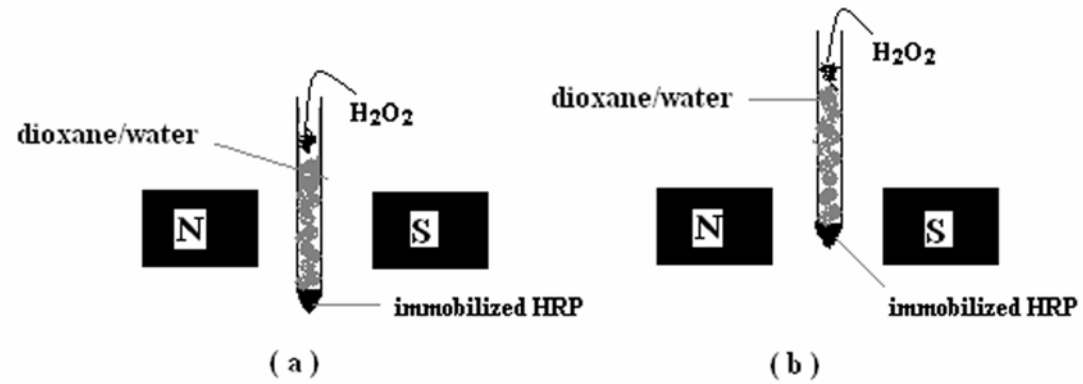

Fig 2.1 Scheme of measurements of ESR spectra with the immobilized enzyme: (a) in and (b) out the magnetic fields

enzyme active centers in the reaction process. The enzyme not only produces free radical in the response, but also provide a field for the free radical, namely a sparse water region lies nearby the enzyme active centers, reaction occur at here . To further confirm the supposition, we utilize paramagnetic technical and immobilized enzyme technology to analysis the response.

Free Radical Detection in Immobilized HRP catalyzed Reaction

HRP immobilized in agar-agar bead put in the capillary vessel base in the paramagnetic meter sample area. Adjust capillary vessel altitude to enable immobilized HRP to be in or out the resonant cavity. In the same experiment condition, ESR spectra were detected in Fig 2.2. Fig 2.2 showed the free radical signal did not examine when the enzyme emigrates outside the resonant cavity. This system does not have any shielding device, therefore it powerfully proved our deduction accuracy.

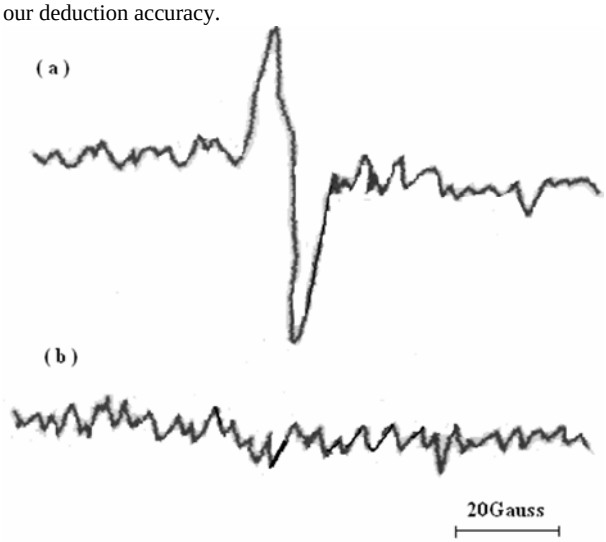

Fig 2.2 The ESR spectra of the solution with the immobilized enzyme (a) in and (b) out the magnetic field.

In Immobilized Enzyme Catalyze phenylphenol polyreaction, we discovered that when add immobilized HRP in reaction solution, the precipitation did not present in the solution, but only observed that the colorless agar-agar bead turned into black (Fig 3). This phenomenon showed the free radical produced by the enzyme catalyze has not diffused into the solution, only happened on enzyme surface, so the product formed adhered to surface of the immobilized HRP (agar-agar bead). The above result may explain that peroxidase catalyzed the phenol polyreaction is different with the general sense chemistry free radical chain reaction. Here, peroxidase participate in each reaction step, the entire reaction is an enzyme catalysis process.

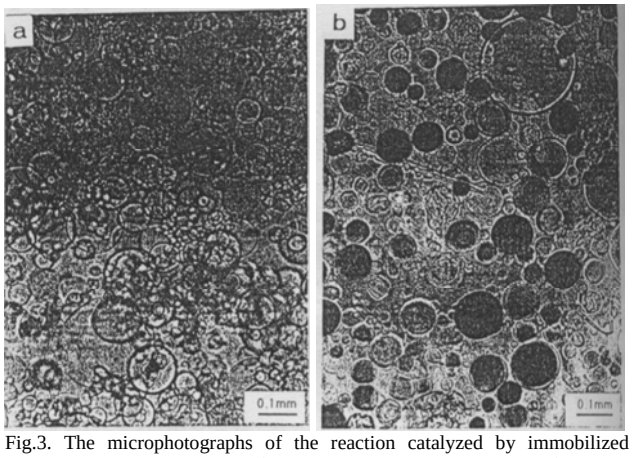

Fig.3. The microphotographs of the reaction catalyzed by immobilized enzymes. The reaction was performed on carrier plate. One drop of reaction solution was put on, which contained immobilized HRP and 0.01mol.l^{-1} p-phenylphenol. The photograph (a) and (b) were taken before and after the addition of 1 μ l, 0.1 mol.l^{-1} H_2O_2 for 10 minutes, respectively.

Although the peroxidase catalyzing hydrogen peroxide or the phenol oxygen free radical cannot free move in the solution, Horseradish peroxidase-mediated polymerization of styrene and methyl methacrylate were succeeded when beta-diketones as initiator in recent years(Kalra *et al* 2000; Singh *et al* 2000). In the reaction process, stoichiometric ratio of hydrogen peroxide and styrene or methyl methacrylate is not equal, the free radical produced by enzyme-catalyzed hydrogen peroxide captured by beta-diketones, subsequently initiated methyl methacrylate or the styrene polymerization. Therefore this kind of polymerization is completely different from peroxidase catalysis phenol compound's polymerization(Silvia *et al* 2008; Wei *et al* 2002).

Conclusions

We investigated the free radical in phenol oxidation process in two designed experimants: a homogeneous reaction and a immobilized HRP catalyzed reaction. The results showed the free radical produced by peroxidase only move on the surface of enzyme, can't free move in solution in both experiment.The phenol polymerization is enzyme reaction process, different from general chemistry free radical chain reaction.

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