Preparation of Cu(II)-Chelated Poly(vinyl alcohol) Nanofibrous Membranes for Catalase Immobilization

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ABSTRACT: Poly(vinyl alcohol) (PVA) nanofibers were formed by electrospinning. Metal chelated nanofibrous membranes were prepared by reaction between Cu(II) solution and nanofibers, and which were used as the matrix for catalases immobilization. The constants of Cu(II) adsorption and properties of immobilized catalases were studied in this work. The Cu(II) concentration was determined by atomic absorption spectrophotometer (AAS), the immobilized enzymes were confirmed by the Fourier transform infrared spectroscopy (FTIR), and the amounts of immobilized enzymes were determined by the method of Bradford on an ultraviolet spectrophotometer (UV). Adsorption of Cu(II) onto PVA nanofibers was studied by the Langmuir isothermal adsorption model. The maximum amount of coordinated Cu(II) (q_m) was 2.1 mmol g^{-1} (dry fiber), and the binding constant (K_l) was 0.1166 L $mmol^{-1}$. The immobilized catalases showed better resistance to pH and temperature inactivation than that of free form, and the thermal and storage stabilities of immobilized catalases were higher than that of free catalases. Kinetic parameters were analyzed for both immobilized and free catalases. The value of $V_{\max}$ (8425.8 μmol mg^{-1}) for the immobilized catalases was smaller than that of the free catalases (10153.6 μmol mg^{-1}), while the K_m for the immobilized catalases were larger. It was also found that the immobilized catalases had a high affinity with substrate, which demonstrated that the potential of PVA-Cu(II) chelated nanofibrous membranes applied to enzyme immobilization and biosensors. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 120: 3291–3296, 2011

Key words: poly(vinyl alcohol); membranes; Cu(II)-chelated; enzymes; kinetics

INTRODUCTION

Nanofibers have gained a great deal of attention in recent decades due to such properties as high surface area per unit mass, excellent structural mechanical strength, remarkable high porosity, and so on. The expanding applications of nanofibers include drug delivery, filtration, tissue engineering, and biosensors. Especially in the area of biocatalysis nanofibers show superiority and distinctive characteristics. Electrospinning is the most common technique for preparing nanofibers, in which a polymer jet is ejected when the electrostatic force applied to the polymer liquid overcomes the surface tension of the polymer solution. The charged jet is elongated and accelerated in the electrostatic field, solvent evaporation, and deposition on a substrate at random.[1]

Enzymes are well known green catalysts which are highly specific and efficient. However, the applications of enzymes are suffering from various problems, e.g., instability, nonreusability, and high-cost.[2] Enzyme immobilization has become an effective way to overcome these limitations to some extent. Insoluble supports can be recycled much more easily than soluble enzymes. At the same time, the multiple-point attachment to the support can restrict the undesirable conformational change of enzyme proteins in practical applications.

Poly(vinyl alcohol) (PVA) is a cheap, nontoxic, hydrophilic, biocompatible synthetic polymer, and there are plenty of hydroxy (OH) groups in it. PVA has been widely used for cell and enzyme immobilization.[3]

Catalases are heme-containing metallo-enzymes, and every catalase protein consists of four subunits, each of them includes ferriporphyrin as a prosthetic group. Catalases are enzymes that decompose hydrogen peroxide (H_2O_2) into water and oxygen. These enzymes are commonly used in various fields,

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including the food, textile, agriculture, and detergent etc. The use of catalases is very effective in the aspects of lower resources and energy consumption.[4]

Enzyme immobilization on the metal chelated support is based on multipoint interactions between chelated metal ions on the support and the imidazole group of histidine, the indole group of tryptophan and the thiol group of cysteine. Most of the transition metal ions such as Ni^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+} can form stable complexes with electron-rich compounds and coordinate molecules containing O, N, and S, for example, amine groups (NH_2), hydroxyl groups (OH), and thiol group (SH).[5]

In this study, PVA nanofibers were electrospun. Then, Cu(II) were incorporated into the hydroxyl (OH) groups of the PVA nanofibers via metal chelation, and PVA-Cu(II) chelated nanofibers were prepared, and the chelated reaction kinetics of PVA nanofibers to Cu(II) were established.

Subsequently, catalases were immobilized onto the metal chelated nanofibers, and the decomposition of H_2O_2 was studied by examining the activity and stability of the immobilized catalases. Additionally, the kinetic parameters for both free and immobilized enzymes were investigated.

EXPERIMENTAL

Materials preparation

Bovine liver catalases (hydrogen peroxide oxidoreduction; EC. 1.11.1.6) were purchase from Sigma. PVA1799 sample with molecular weight of 84,000–89,000 was obtained from Shanghai Kanghu Chemical. Copper dichloride, hydrogen peroxide, formic acid, and Coomassie brilliant blue (G250) for the Bradford protein assay was purchase from Shenyang Sinopharm Chemical Reagent. Hydrogen peroxide (30%) and the ingredients of phosphate buffer solution such as NaCl, KCl, KH_2PO_4, K_2HPO_4 were of analytical grade and used as received. Water used in all experiments was de-ionized.

Electrospinning

Electrospinning was carried out to fabricate PVA nanofibers. PVA was dissolved in formic acid (with a purity of 88%) at room temperature with stirring. PVA solutions were placed in syringes (content of 20 mL) with a 0.7-mm diameter spinner jet, and the solution flow rate was controlled by a microinfusion pump (WZ-50C2, Zhejiang, China). The high-voltage supplier (DW-P503-4AC, Tianjin, China) was used to connect metal needles and the grounded collector for forming electrostatic fields. The grounded collection roller covered with the aluminum foil was used to collect the nanofibers. The parameters of electrospinning were set as follows: voltage 14 KV, solution flow rate 0.3 mL h^{-1}, and collecting distance between the syringe needle tip and the grounded collector 14 cm. Nanofibers were collected by roller for 24 h.

Chelation of Cu(II) on PVA nanofibers

Chelation of Cu(II) with PVA nanofibers were prepared as follows: 0.2 g of the PVA nanofibers with an average diameter of about 160 nm were put in flasks, each of them containing of 20 mL copper dichloride solution (395.16, 318.44, 237.34, 159.53, 78.59, 39.38, 23.75, 7.81 m*M* Cu(II) concentration at pH 7.0). The flasks were stirred at 20°C for 24 h. The nanofibrous membranes adsorbed Cu(II) were washed with deionized water. The amount of adsorbed Cu(II) was calculated by using the concentrations of the Cu(II) in the initial solution and in resulting solution. The Cu(II) concentration was determined by atomic absorption spectrophotometer (AAS). The PVA-Cu(II) nanofibrous membranes were dried in air and were stored as nanofibrous membranes.

Langmuir isothermal adsorption model, which applies to many polymer binding system.

$$q = \frac{q_m K_l C_e}{1 + K_l C_e} \quad (1)$$

where q_m is the maximum amount of coordinated Cu(II), K_l is the binding constant, C_e is the equilibrium constant. To get the best values for the binding parameters from the experimental results, the eq. (1) can be rearranged as below:

$$\frac{1}{q} = \frac{1}{q_m K_l C_e} + \frac{1}{q_m} \quad (2)$$

The reaction kinetics was studied by the Langmuir isothermal adsorption model.

Immobilization of enzymes on metal chelated nanofibrous membranes

PVA-Cu(II) chelated nanofibrous membranes were immersed in 20 mL of catalases solution (0.3 mg mL^{-1}) in 50 m*M* phosphate buffer solution pH 7.0 for 4 h at 20°C in shakers while stirring continuously. Then, the nanofibrous membranes were removed from solution and rinsed with the same phosphate buffer solution until no soluble protein was detectable. Enzymes concentration was determined by the method of Bradford, the amount of the catalases was determined spectrophotometrically according to absorbance of Coomassie brilliant blue

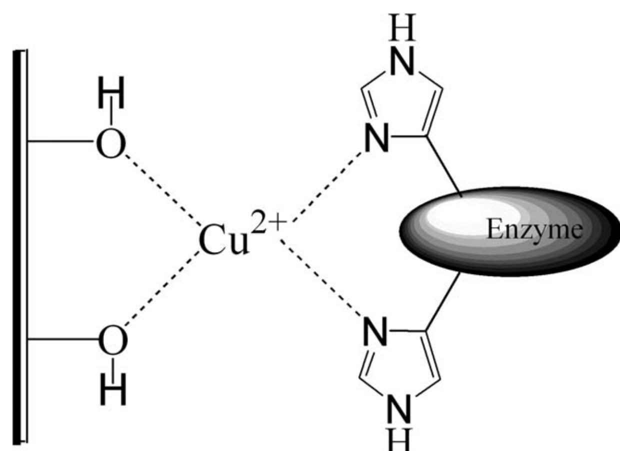


Figure 1 Schematic of PVA-Cu(II) chelated nanofibrous membrane immobilized by catalase.

and catalases at 595 nm, and the amount of bound enzymes were calculated as:

$$Q = \frac{(C_0 - C)V}{m} \tag{3}$$

Here, Q is the amount of catalases bound onto unit mass of nanofibrous membranes (mg g^{-1}), C_0 and C are the initial and equilibrium enzymes concentrations in the solution (mg mL^{-1}), V is the volume of the catalases solution, and m is the mass of the nanofibrous membranes.

Fourier transform infrared (FTIR) spectra were recorded using a NICOLET NEXUS 470 FTIR spectrometer. Schematic of PVA-Cu(II) chelated nanofibrous membrane immobilized by catalase is presented in Figure 1. Cu(II) can chelate with hydroxyl groups on PVA nanofibers and the imidazole groups on catalases by coordination bond, and enzyme immobilized onto Cu(II)-PVA matrix.

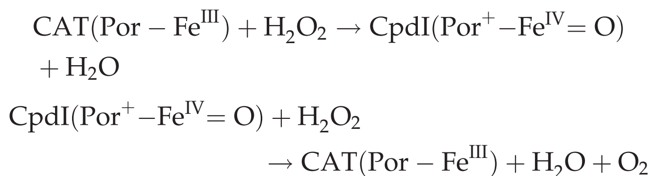
Activity assays of the free and immobilized enzyme

The activity of the free and immobilized catalases was determined spectrophotometrically by measurement of decrease in the absorbance change of hydrogen peroxide at 240 nm. Catalases immobilized chelated nanofibrous membranes were mixed with 20 mL of hydrogen peroxide solution (100 mM) in 50 mM phosphate buffer solution (pH 7.0). The reaction was kept at 35°C for 3 min and the specific activity of enzyme was calculated by the following formula:

$$v = \frac{\Delta A \times V}{T \times K \times Ew} \tag{4}$$

where ΔA is the absorbance decrease of the solution at 240 nm, V is the volume of the hydrogen peroxide solution, T is the time of reaction, K is the molar extinction coefficient of hydrogen peroxide at 240 nm, and E_w is the amount of enzyme.

The catalytic mechanism of catalases is presented as follows[6]:



$$\mathrm{CAT(Por - Fe^{III}) + H_2O_2 \rightarrow CpdI(Por^{+}-Fe^{IV}{=}O) + H_2O}$$

$$\mathrm{CpdI(Por^{+}-Fe^{IV}{=}O) + H_2O_2 \rightarrow CAT(Por - Fe^{III}) + H_2O + O_2}$$

Determination of kinetic parameters

The effect of hydrogen peroxide concentration on the activity was tested, and $V_{\max}$ and K_m values of free and immobilized enzyme were calculated by the Lineweaver-Burk plots.

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}} \tag{5}$$

where K_m is the Michaelis constant, v and $V_{\max}$ represent the initial and maximal rate of the reaction, and $[S]$ is the concentration of the hydrogen peroxide. Kinetics parameters for immobilized and free enzymes were investigated at 35°C and the concentrations of hydrogen peroxide ranged from 20 to 200 mM (pH 7.0).

Dependence of temperature and pH on the activity of immobilized and free enzyme

The immobilized and free enzymes were mixed with 20 mL of hydrogen peroxide solution (100 mM) in 50 mM phosphate buffer (pH 7.0) respectively, and the temperatures ranging from 15 to 65°C. The optimum pH values of free and immobilized enzymes were investigated at 35°C for 3 min. Phosphate buffer solution (pH 5.0–8.5) was used for pH dependence study.

Storage and operational stability of the free and immobilized enzyme

The stability of free and immobilized enzyme at storage was measured by calculating their activity retention during 20 days at 4°C in 50 mM phosphate buffer solution (pH 7.0), using 2-day intervals, then a sample was removed and determined for enzyme activity as described above. The loss of activity of the immobilized enzyme after use was checked. After each reaction run, the enzyme was removed and washed with 50 mM phosphate buffer solution (pH 7.0) to remove any residual substrate within Cu(II)-PVA matrix; enzyme activity was then detected under optimum conditions.

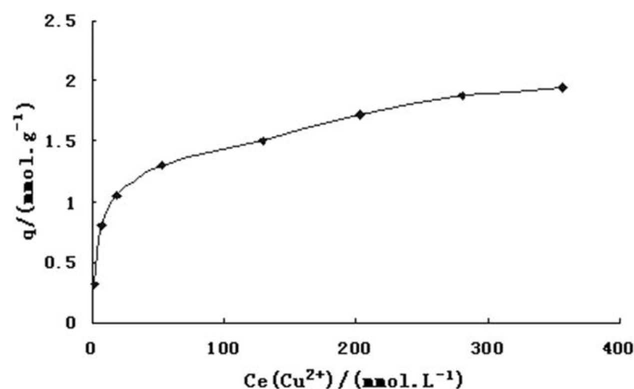


Figure 2 Relationship between q and C_e.

The thermal stability of the free and immobilized enzyme

The thermal stability of the free and immobilized enzyme was investigated by measuring the residual activity of the enzyme exposed at various temperatures (20–55°C) in phosphate buffer solution for 4 h. Enzyme activity was detected under optimum conditions as given above.

RESULTS AND DISCUSSION

Adsorption isotherm of Cu(II) on PVA nanofibers

According to experimental results, plots of q versus C_e and $1/q$ versus $1/C_e$ are shown in Figures 2 and 3.

It is clearly observed from Figure 2 that adsorption of Cu(II) increased significantly with the rise in concentration from 7.81 to 395.16 mM and then gradually leveled off. The adsorption equilibrium data could be interpreted by the Langmuir absorption equation. The basic assumption of the Langmuir theory is once a metal ion occupies a reaction site, then no further adsorption occurs at that location.[7] The initial increase in Cu(II) adsorption might be due to many available chelating hydroxyl groups in PVA nanofibers, and reached saturation gradually with the increase in concentration of Cu(II). The reaction kinetics parameters could be calculated through straight line slope and ordinate intercept of plots. The saturated coordinate capacity of PVA to Cu(II) was 2.1 mmol g^{-1} (dry fiber), and the reaction rate constant K_l was 0.1166 L mmol^{-1}.

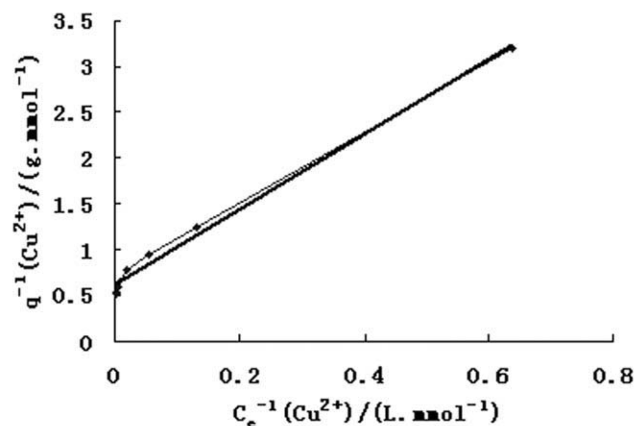


Figure 3 Relationship between $1/q$ and $1/C_e$.

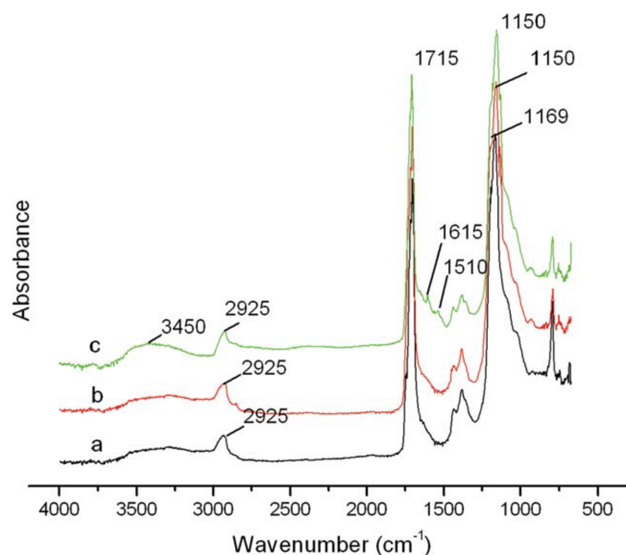


Figure 4 FTIR spectra of (a) PVA, (b) PVA-Cu(II), and (c) PVA-Cu(II)-catalase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FTIR spectra

The FTIR spectrum of the PVA [Fig. 4(a)] shows the following characteristic peaks: 2925 cm^{-1} C—H stretching vibration of —CH_2. The wide band at 3550–3150 cm^{-1} corresponded to the O—H stretching vibration. The bands at 1169 cm^{-1} were assigned to the C—O stretching vibration, and the bands at 1715 cm^{-1} indicated that residual carboxyl groups in a trace amount of formic acid.

Adsorption band of C—O at 1165 cm^{-1} [Fig. 4(a)] moved to 1150 cm^{-1} [Fig. 4(b,c)] due to the decrease of bond force constant coupled with decrease of the electron density, which indicated that the hydroxyl groups of PVA coordinated with Cu(II).[8]

The characteristic stretching vibration amide I and amide II absorption bands at 1615 and 1510 cm^{-1} are shown [Fig. 4(c)], respectively. Moreover, the wide band of 3600–3150 cm^{-1} corresponded to the N—H and O—H stretching vibration. These indicated the immobilization of the enzyme to the matrix.

Kinetic parameters of immobilized and free catalases

The kinetics parameters V_{max} (maximum reaction rate) and K_m (Michaelis-Menten constants) are shown in Table I. According to data, K_m of the

TABLE I
Kinetic Parameters of the Immobilized and Free Enzymes and the Amount of Bound Enzymes

	Amount of bound enzymes (mg g^{-1} fibers)	Specific activity (U mg^{-1})	K_m (mM)	V_{max} (μmol mg^{-1} protein min)
Free catalase	–	2120.0	42.3	10153.6
Immobilized catalases	62.3	923.2	67.5	8425.8

immobilized catalases was higher than that of free catalases. At the same time, V_{max} of the immobilized catalases was smaller than that of free catalases. These data indicated that affinity between immobilized catalases and substrate decreased comparing with free catalases. It might be affected by the structural changes of immobilized enzymes, with inclusion of less accessibility between substrate and active points of immobilized enzyme caused by space barriers of the supports.[9–11]

Effect of temperature and pH on the enzyme activity

The effect of temperature on the activity of free and immobilized is depicted in Figure 5. It is obviously revealed that the initial relative activity was increased with the increase in temperature and then decreased as the temperature was further increased for both immobilized and free catalases. The optimum temperatures for the immobilized and free catalases were observed at $\sim$ 40 and 35°C, respectively. At higher temperature range, the immobilized catalases exhibited higher stability than the free one, and it showed the antithermal property of the immobilized enzymes. The multipoint interactions between enzymes and PVA-Cu(II) support might reduce the degree of freedom of the spacial structure of enzyme, protecting it to some extent from deactivation at the high temperature.

Changes in pH values could affect the enzyme conformation and the dissociation degree of substrate and coenzyme, and thus affect the binding and catalysis between the enzyme molecules and substrate, only at a specific pH value, the most appropriate for enzyme and substrate to be combined, and the occurrence of a catalyst. The effect of pH on the activity of free and immobilized catalases is shown in Figure 6. According to experimental results, the significant shift of optimal pH was not observed. The optimal pH value was found at about 7.0 for free and immobilized enzyme, but the pH profiles of the immobilized enzyme were much broader than that of the free enzyme. The residual relative activity of the immobilized catalases was higher than that of the free catalases in the pH range between 5.0 and 8.5. It was found that the immobilized enzymes showed less sensitivity to pH than the free one, which was probably because of the production of oxygen, forming foams, and causing slight diffusion limitations on the surface of nanofibrous membrane.[12]

Storage and operational stability

The storage stability of immobilized and free enzymes was also studied and the results are presented in Figure 7. Both the relative activities of free and immobilized catalases were dropped down with the increase in storage time. The immobilized catalase lost 52% of its activity within 20 days, and the free catalase lost 81% of its activity during the same period. The result indicated that the

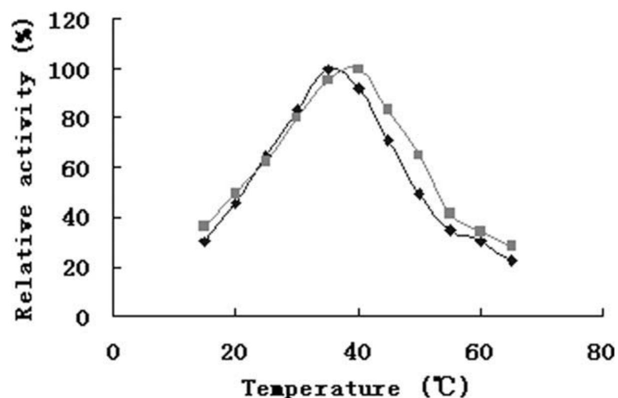


Figure 5 Effect of temperature on the (■) immobilized and (◆) free catalases.

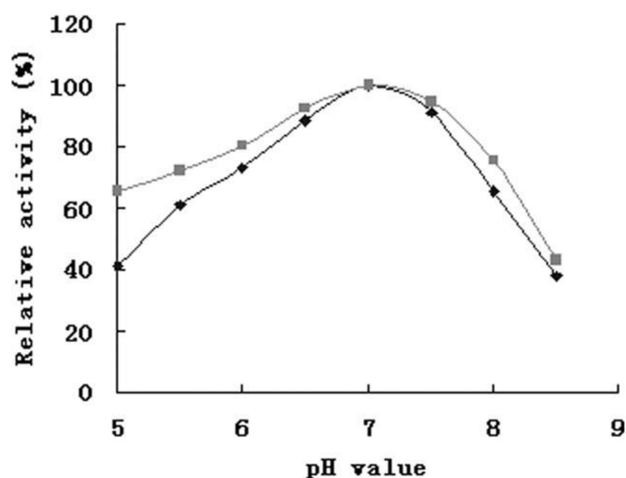


Figure 6 Effect of pH on the (■) immobilized and (◆) free catalases.

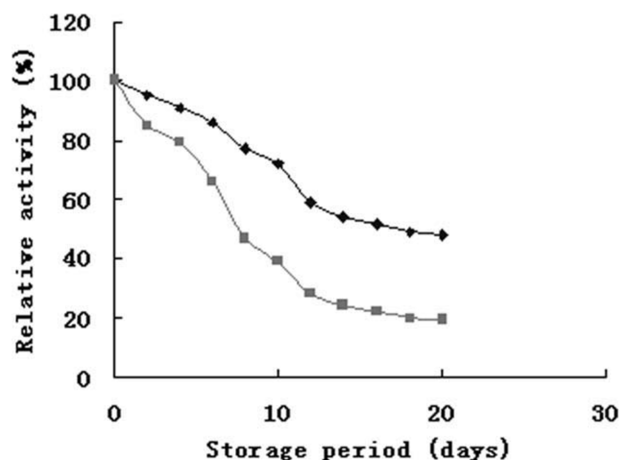


Figure 7 Storage stability of (◆) immobilized and (■) free catalases.

immobilized catalases storage stability was much better than that of free catalases, which might be attributed to the immobilization of enzyme to a matrix that limited their freedom to undergo conformational changes resulting in increased stability toward denaturation. The decrease of catalase was explained as a time-dependent natural loss.[13] In general, enzyme in aqueous solution is not stable during storage; storage stability is an important advantage of immobilized enzymes.

According to the effect of repeated use on activity of immobilized catalase, the residual activity of the immobilized enzyme after five-time-use is about 75% of their activity, which shows its potential value in actual application.

Thermal stability

The thermal stability of immobilized and free enzyme is shown in Figure 8. Both relativity activities of the free and immobilized enzyme were decreased gradually with the increase of temperature of phosphate buffer solution for preincubation. At 55°C the immobilized enzyme retained 42% of the initial activity, while the free catalases lost 88% of the initial activity after 4-h preincubation in phosphate buffer solution. The rate of thermal inactivation of immobilized catalase was lower than that of free catalase. The conformational flexibility of enzyme could be affected by immobilization of catalase on the surface of metal chelated nanofibrous membrane, multipoint attachment between immobilized enzyme supports was supposed to maintain the tertiary structure of enzyme, and caused an increase in enzyme rigidity which was reflected by increase in stability toward denaturation by raising the temperature.[14]

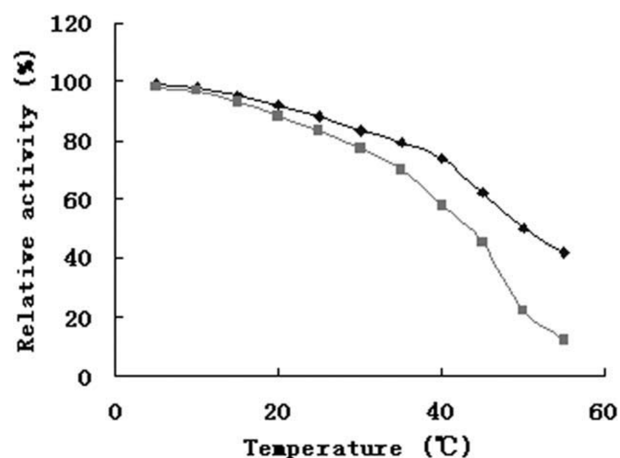


Figure 8 Thermal stability of (◆) immobilized and (■) free catalases.

CONCLUSIONS

Electrospinning was carried out to fabricate PVA nanofibers, and chelated nanofibrous membranes were prepared by reaction between PVA nanofibers and Cu(II) solution. The maximum amount of coordinated Cu(II) and the binding constant were calculated. Catalases were successfully immobilized on the PVA-Cu(II) chelated nanofibrous membranes, the kinetic parameters of catalases were calculated. At the same time, the effects of pH and temperature on the immobilized and free enzymes activity were studied. The immobilized catalases showed significantly improved storage and thermal stability.

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