Immobilization of Horseradish Peroxidase by Entrapment in Natural Polysaccharide

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ABSTRACT: Horseradish peroxidase (HRP), which catalyzes oxidation reduction reactions of large number of substrates, was entrapped in K-carrageenan beads using polyethyleneimine as hardening agent. The heat and storage stability was found to be better for entrapped horseradish peroxidase than free enzyme. The entrapped enzyme showed 50% retention of its activity after 4 cycles. Effective diffusion coefficient for diffusion of hydroquinone into K-carrageenan beads was found to be 0.27×10^{-10} m^2/s during enzyme-catalyzed oxidation of hydroquinone. Kinetic parameters calculated from Lineweaver–Burke plots were observed to be $K_m = 8 \times 10^{-5}$ and $V_{max} = 1.53$ for free enzyme, and $K_m = 8.3 \times 10^{-5}$ and $V_{max} = 2.18$ for entrapped enzyme when enzyme concentration was kept constant and $K_m = 4 \times 10^{-11}$ and $V_{max} = 0.45$ for free enzyme and $K_m = 4.5 \times 10^{-11}$ and $V_{max} = 0.58$ for entrapped enzyme when substrate concentration was kept constant. This indicates that there is no conformational change during entrapment.
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Key words: horseradish peroxidase; entrapment; K-carrageenan; oxidation; hydroquinone

INTRODUCTION

Enzymes as biocatalyst have been widely used in many biological reactions. Free enzymes suffer from certain disadvantages that are overcome by immobilization. The immobilization of enzymes at the interface in a heterogenous system is currently an active area in biotechnology. Immobilized enzymes exhibit higher stability and enable a continuous conversion process with good product recovery and minimal loss of enzyme activity. Horseradish peroxidase (HRP) (E.C.1.11.1.7) belongs to the group of heme proteins. Peroxidase catalyses dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones, hydroquinoid amines, and especially benzidine derivatives. Peroxidase can be determined from the decrease in the concentration of H_2O_2 or in the hydrogen donor or from the formation of the oxidized compound.[1] The conventional methods of enzyme immobilization include physical adsorption, entrapment, and covalent binding of enzyme on various supports.

Peroxidase has been immobilized on various natural and synthetic supports. Schell et al.[2] immobilized horseradish peroxidase on cyanogen bromide activated cellulose. They observed that the optimum pH for the immobilized enzyme activity remained unchanged. The enzyme did not show any loss of activity after repeated use. The storage of immobilized horseradish peroxidase showed 50% loss in the enzyme activity after 15 days at room temperature. Horseradish peroxidase was immobilized on AH-Sepharose 4B by covalent binding and on poly(AAm) by entrapment by Berezin et al.[3,4] They observed that the covalently bound enzyme did not show any change in K_m value but the V_{max} values decreased to 2-fold when *o*-dianisidine was used as substrate. In case of enzyme entrapped in poly(AAm), they found that the entrapped enzyme had less thermal stability than free enzyme D'Angiuro et al.[5–7] prepared graft copolymers of natural polysaccharides such as cellulose, sepharose, sephadex, and starch using glycidylmethaacrylate. They found that there was no change in kinetic parameters of free and enzyme immobilized through entrapment or covalent binding. Natural polysaccharides agarose and sepharose activated with cyanogen bromide and separon HEMA activated with epichlorohydrin were used for immobilization of horseradish peroxidase by Taylor.[8] Better retention of enzyme activity after immobilization was reported at pH 7–9. Use of horseradish peroxidase immobilized on chitosan beads activated with glutaraldehyde for the determination of traces of H_2O_2 in environmental samples was reported by Sakuragawa.[9] Rao et al.[10] immobilized horseradish peroxidase through covalent binding to polystyrene beads activated through water-soluble carbodiimide. The kinetic parameters were reported to be remained unaltered for free and immobilized enzyme. The beads were reported to retain

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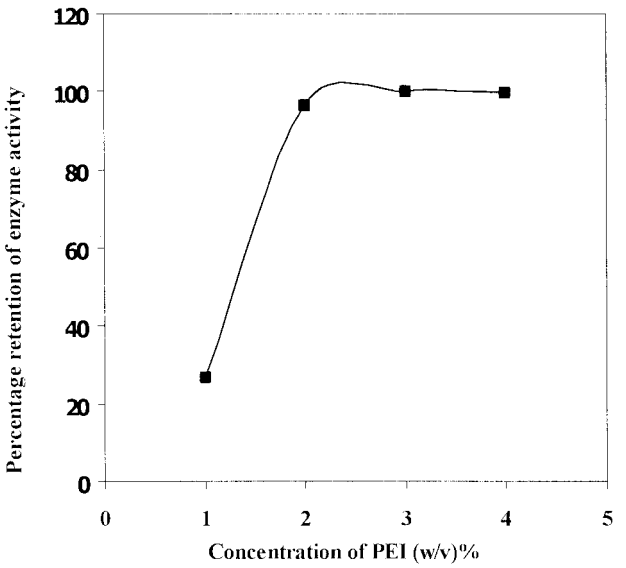

Figure 1 Effect of PEI concentration on the retention of enzyme activity at 35°C, pH 6 for 1 h.

81% of initial enzyme activity after one month when stored at 5°C. Horseradish peroxidase was immobilized by Thibault[11] on aliphatic amino silica activated with glutaraldehyde and aromatic amino silica activated with nitrite. The horseradish peroxidase immobilized on aliphatic amino silica was found to be superior to free as well as that of immobilized on aromatic amino silica. It showed more resistance to 10–30% ethonalic solutions. Horseradish peroxidase was immobilized on microspheres prepared by dispersion polymerization of HEMA and EDMA by Horak.[12] Covalent coupling was carried out through hydrazide formation. Only 7.3 μg of enzyme per gram was reported to be bound without significant loss in enzyme activity. The immobilized horseradish peroxidase showed 97% activity after storage for 23 days at 5°C.

K-Carrageenan is a marine polysaccharide obtained from the red algae *Rhodophyceae.* Chao et al.[13] in 1985 used K-carrageenan for cell immobilization, and found that the beads containing cells treated with polyethyleneimine (PEI) showed better temperature and abrasion resistance, and used them for evaluation of activity of invertase, lactase, and glucose isomerase in a packed bed reactor. The apparent half lives were found to be 108, 39, and 64 days, respectively, for immobilized invertase, lactose, and glucose. For the continuous production of L-amino acid from DL-amino acids, amino acylase immobilized in K-carrageenan was used by Chibata et al.[14] Better pH, temperature, and operational stability compared to free enzyme was reported by Wijffels et al.[15] when the cell of a pure strain of a dentrifying bacteria was immobilized in K-carrageenan. However, to our knowledge there are no reports on the use of K-carrageenan for the immobilization of horseradish peroxidase. Hence an attempt has been made to optimize the conditions for the entrapment of horseradish peroxidase in K-carragennan and its use in oxidation of hydroquinone.

EXPERIMENTAL

Materials

HRP (E.C.1.11.1.7), of strength 200 U mg^{-1}, was obtained from Sigma Chemicals Co., U.S.A. Pyrogallol, hydroquinone and H_2O_2 30% (w/v) were from E. Merck, Mumbai, India, and potassium dihydrogen *ortho*-phosphate (KH_2PO_4) from Spectro Chem India were used as received K-Carrageenan (1-3-β-D-galactopyranose-4-sulfate-(1-4)-3,6 anhydro-α-D-galactopyranose-(1-3) was received from Central Salt and Marine Chemical Research Institute, Bhavnagar, India.

Assay method of horse radish peroxidase

The activity of free and immobilized enzyme was determined by using pyrogallol as a substrate as described by Worthington.[16] The enzyme-catalyzed reaction was monitored in 0.1*M* phosphate buffer of pH 6, using 0.147*M* 30% (w/v) of H_2O_2 and 5% (w/v) pyrogallol solution at 35°C. The amount of liberated purpurogallin was estimated spectrophotometrically at 420 nm.

Method of entrapment of HRP

The entrapment of HRP in K-carrageenan bead was done by modifying the method given by Jose et al.[17]

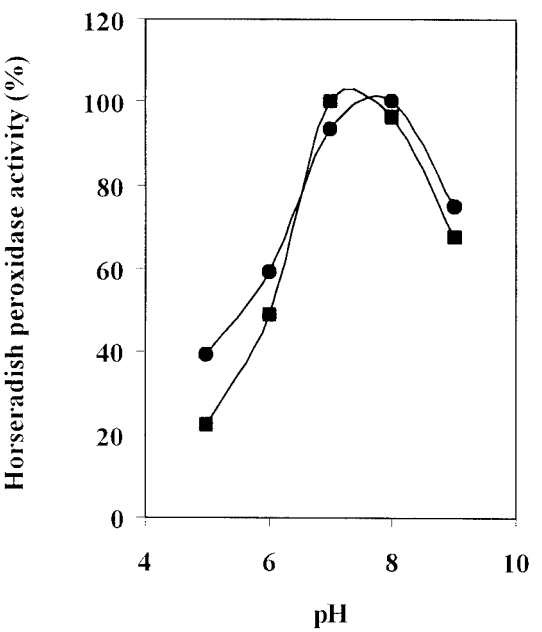

Figure 2 Effect of pH on the activity of free and immobilized HRP at 35°C; free enzyme (■), entrapped HRP (●).

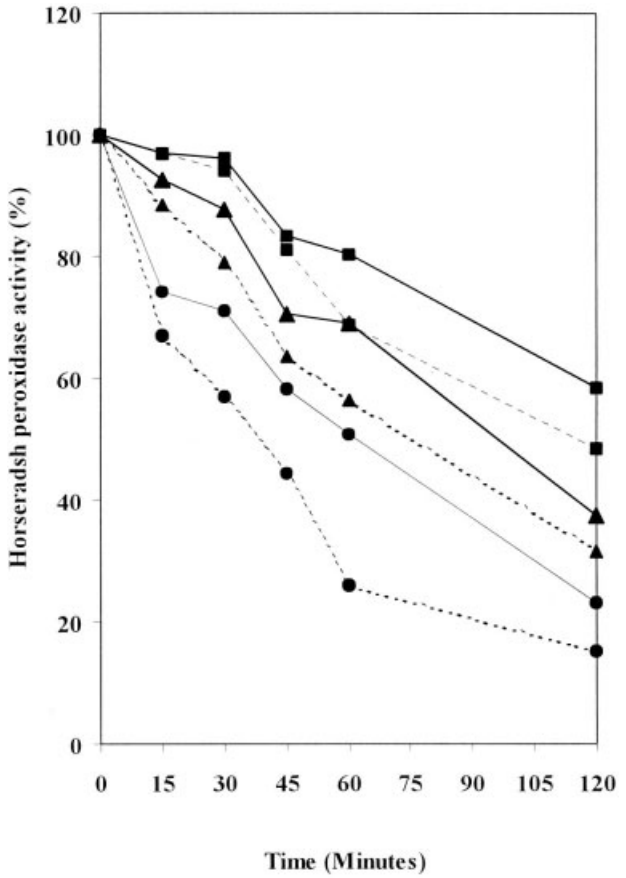

Figure 3 Effect of temperature on the activity of free and immobilized HRP at pH 6 at 35°C (■), 45°C (▲), and 55°C (●); free enzyme (—), entrapped enzyme (—).

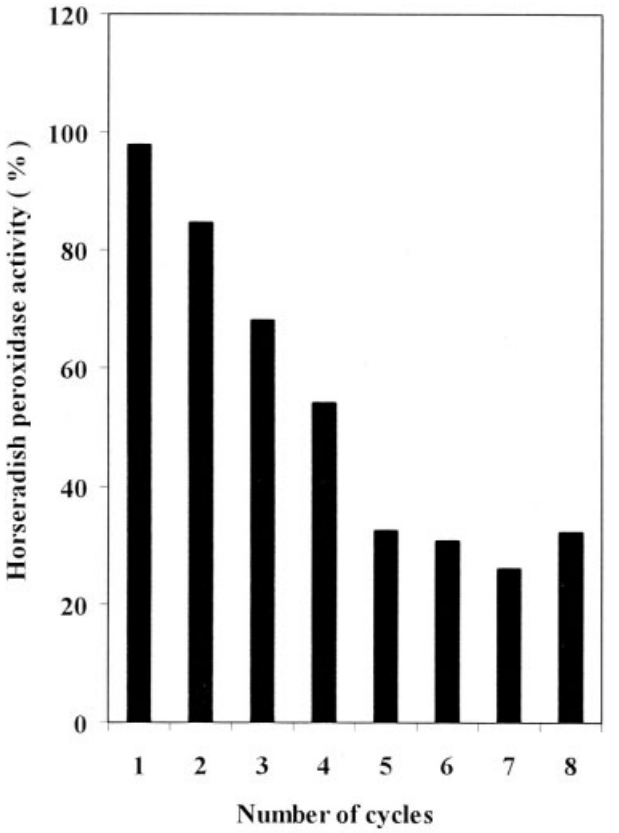

Figure 4 Reusability of entrapped enzyme at 35°C and pH 6.

K-Carrageenan (3% w/v) solution was prepared in water at 60°C and cooled below 35°C for 30 min To 2 mL of this solution 1 mg of HRP in 1 mL of water was added and mixed thoroughly. The resulting solution was dropped in 2% w/v PEI solution using syringe. The beads were allowed to harden in PEI for 2 h and then washed several times with water for removal of PEI.

Effect of polyethyleneimine concentration

The effect of PEI concentration on bead hardening and retention of entrapped enzyme activity was studied by using 1–4% PEI solution. The K-carrageenan beads containing enzyme were prepared as described earlier and they were allowed to harden in 1–4% w/v solution of PEI for 2 h. The protein content in supernatant liquid was calculated according to Lowry's method. From the differential method the protein content in the beads was calculated. The activity of entrapped enzyme was calculated as per the procedure described by Worthington.[16] From this percentage retention of activity of entrapped enzyme was calculated with respect to protein content in the beads. The effect of hardening time on enzyme retention in the beads was studied by varying the time of contact from 1 to 4 h.

TABLE I
Effect of Temperature and Time on Deactivation of Enzyme

	Thermodeactivation constant × 10^3 at					
	35°C		45°C		55°C	
Time in min	Entrapped	Free	Entrapped	Free	Entrapped	Free
15	0.206	0.206	0.510	0.811	1.900	2.600
30	1.330	2.060	4.380	7.890	11.400	18.700
45	4.060	4.810	7.730	10.000	11.900	18.700
60	3.630	6.250	6.210	9.500	11.200	22.500
120	4.480	6.025	8.160	9.500	12.200	25.700

TABLE II
Effect of Enzyme Concentration on Retention of Entrapped Enzyme Activity

Enzyme taken for entrapment in mg (U)	Total protein content in the beads mg	Active Protein in beads mg	Percentage retention of enzyme activity in beads
1 (200)	0.125	0.0429	34
3 (600)	0.893	0.260	29
5 (1000)	1.469	0.551	38
7.5 (1500)	1.907	0.609	32

Dependence of pH

The pH activity profile of free and immobilized HRP was constructed by incubating the free and immobilized enzyme at 35°C for 15 min in the buffer solutions of different pH and using pyrogallol as substrate. The purpurogallin produced was estimated at 420 nm. From the calibration plot the activity of enzyme was determined.

Thermal stability

The thermal stability was checked by incubating free and immobilized enzyme in the buffer of optimum pH at various temperatures (35–55°C) for different time intervals. The thermodeactivation constant (K_d) was calculated by using the following equation[18]:

$$\ln A_t = \ln A_0 - K_d(t) \quad (1)$$

where A_0 is initial activity of enzyme, and A_t the activity after heat treatment for t min.

Storage stability

The free and immobilized enzymes were stored at 35°C and the percentage residual activity was determined at different time intervals.

Reusability of entrapped HRP

The reusability of immobilized HRP was checked by using the same beads with fresh aliquots of substrate (0.3 mL 5% w/v) and determining the retention of enzyme activity. The process was continued until the beads showed 50% loss in enzyme activity.

Determination of kinetic parameters

Enzymatic reactions are controlled by substrate and enzyme concentration. Therefore the kinetic parameters such as maximum reaction velocity (V_{max}) and Michaelis constant (K_m) were calculated by varying the substrate concentration from 0.39 to 1.9 mole/L and keeping the enzyme concentration constant at 4 $\times 10^{-7}$ mole/L and also by varying enzyme concentration from 2.5×10^{-7} to 12.5×10^{-7} mole/L and keeping substrate concentration 0.39 mole/L and using Lineweaver–Burke plots.

Oxidation of hydroquinone

In 1967 Taylor et al.[19] used free HRP for the manufacture of *p*-benzoquinone from hydroquinone. Immobi-

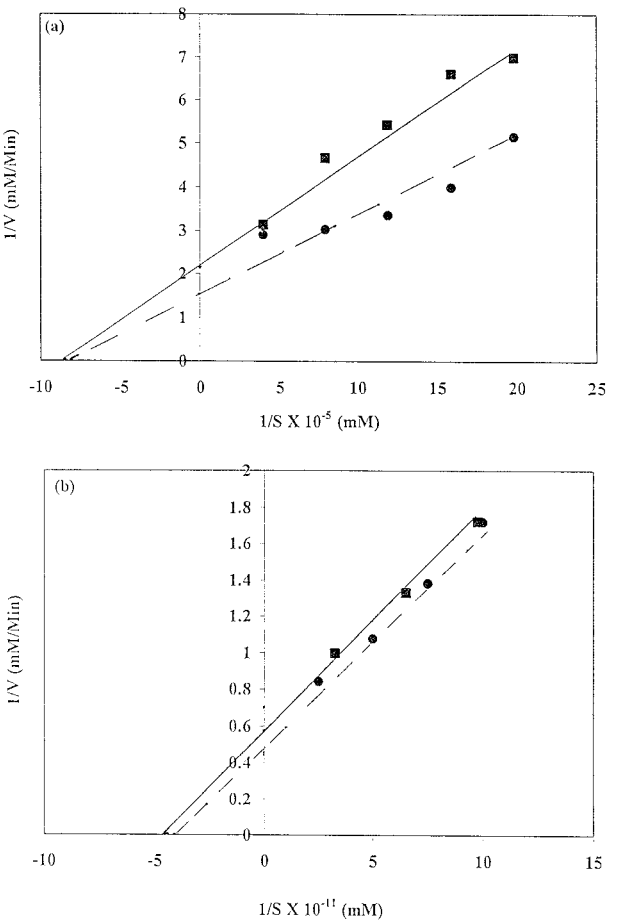

Figure 5 Lineweaver–Burke plots at 35°C, pH 6, and 1 min reaction time; free HRP (—), and entrapped HRP (—). (**a**) Enzyme concentration 4×10^{-12} mole/L; pyrogallol concentration 0.39 to 1.9 mole/L. (**b**) Pyrogallol concentration 0.39 mole/L; enzyme concentration 2.5×10^{-7} to 12.5×10^{-7} mole/L.

TABLE III
Kinetic Parameters for Free and Entrapped HRP

	Free enzyme		Entrapped enzyme	
*Conditions[a]	K_m (mM)	V_{max} (mM/min)	K_m (mM)	V_{max} (mM/min)
When substrate conc. is 0.39–1.9 mole/L and enzyme concentration is 4×10^{-12} mole/L	8×10^{-5}	1.53	8.3×10^{-5}	2.18
When enzyme concentration is 2.5×10^{-7} to 12.5×10^{-7} mole/L and substrte con.0.39mole/litre.	4×10^{-11}	0.25	4.5×10^{-11}	0.58

[a] At 35°C, pH 6, for 1 min.

lized enzyme has inherent advantages. However, though immobilized whether HRP has been used in the oxidation of phenol from industrial effluent[20,21] has not been reported for the production of *p*-benzoquinone through hydroquinone oxidation. The chemical route of this process, being exothermic, needs to be carried out at 5°C in the presence of catalyst. Hence we have tried the reaction by using immobilized HRP. The kinetics of the reaction was studied by monitoring the reaction at various time intervals. The effect of enzyme concentration and pH on oxidation was also studied.

Diffusion experiments

Owing to the diffusional limitation of the substrate in the bead during oxidation of hydroquinone, we have tried to study the diffusion of substrate into the K-carrageenan beads. The diffusion experiments were carried out by using the procedure given by Jovetic et al.[22] The effective diffusion coefficient (D_e) is the parameter that mathematically describes the rate of transport per unit driving force. Diffusion experiments were carried out at 35°C. The 0.4 mM solution of hydroquinone in a closed container was stirred for 2 h to avoid external mass transfer resistance. The known amount of beads of 1.5–2 mm diameter were placed in a known volume of hydroquinone solution in a closed container. The volume change of the beads due to the diffusion of solution was measured and was used for determination of diffusion coefficient. Almost no change in volume of beads was observed after 6 h.

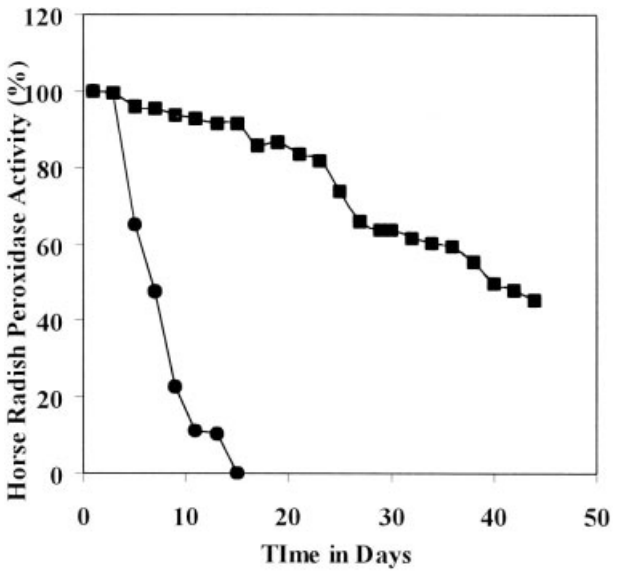

Figure 6 Storage stability of free and entrapped enzyme at 35°C, and pH 6; free enzyme (●), and entrapped HRP (■).

RESULTS AND DISCUSSION

Effect of PEI concentration

Potassium chloride has been widely used as a curing agent for hardening of K-carrageenan beads.[14] However, we have observed that the mechanical strength of the beads formed in KCl is not desirable. Chibata[23] has used PEI as a curing agent and claimed it a better hardening agent. Hence we have used 1–4% PEI solution for curing of K-carrageenan beads. From the results given in Figure 1 it has been observed that desired hardening of beads and the retention of entrapped enzyme activity is achieved at 2% w/v PEI concentration. Further increase in the PEI concentration did not show any change in the entrapped enzyme activity.

Effect of pH on enzyme activity

Enzymatic reactions are pH dependent. Hence pH profile for the free and entrapped enzyme was stud-

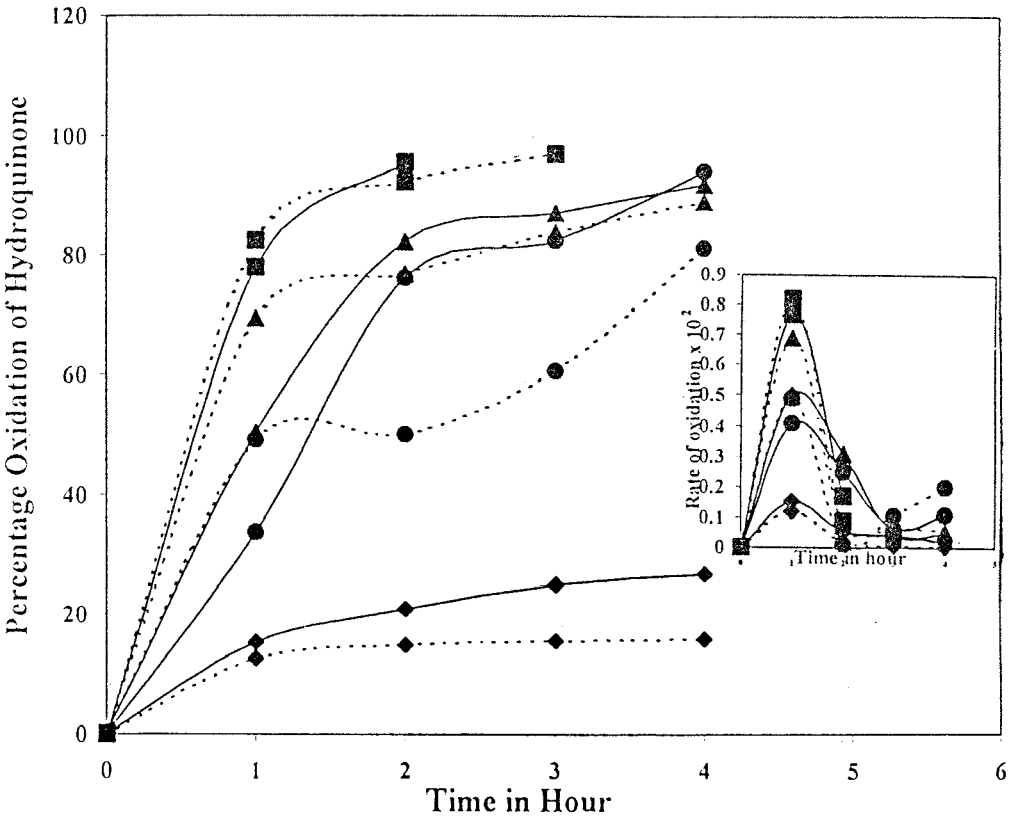

Figure 7 Effect of concentration of hydroquinone on the rate of oxidation at 35°C and enzyme concentration 50 μg; free enzyme (—) and entrapped enzyme (—); 1 m*M* (■), 2 m*M* (▲), 3 m*M* (●), and 4 m*M* (◆).

ied. From Figure 2 it was observed that free enzyme exhibits maximum activity at pH 8 whereas entrapped enzyme shows maximum activity at pH 7. As the enzyme is immobilized through entrapment, it is not expected to observe any conformational changes. However, a slight shift in pH towards acidic side on entrapment can be attributed to the anionic nature of K-carrageenan polysaccharide.

Thermal stability

Free enzymes suffer with the poor thermal stability. Immobilization of enzymes generally improves thermal stability, which is desirable for the reactions to be carried out at elevated temperature. From the results illustrated in Figure 3, it is observed that entrapped enzyme has better thermal stability. The thermodeactivation constants are given in Table I. At all temperatures and times, entrapped enzyme showed better thermal stability. This can be attributed to a protective sheath formed around the enzyme during entrapment, which helps in decreasing the thermal impact and hence deactivation effect.

Effect of enzyme concentration

The effect of enzyme loading on 3% K-carrageenan beads was studied by using 200–1500 U enzyme solution. It was observed that with the increasing concentration of the enzyme, the amount of protein and activity of enzyme in beads increases but the percentage retention of enzyme activity remains constant (Table II).

Reusability of entrapped enzyme

Free enzyme suffers from a drawback of nonreusability and this drawback is overcome by immobilizing it. Here we have observed that immobilized enzyme retained 50% activity after 4 cycles (Fig. 5) and about 25% of activity after 8 cycles, thus showing an advantage over free enzyme and making it useful in a large number of applications. The turnover number of entrapped enzyme was observed to be 51.6×10^6.

Kinetic parameters

The kinetic parameters (K_m) and (V_{max}) for HRP entrapped in K-carrageenan were calculated from Lineweaver–Burke plot of 1/V vs 1/S [Fig. 5(a,b)]. The results are given in Table III. The results show that there is no change in K_m and V_{max} for free and entrapped enzyme when substrate concentration or enzyme concentration is kept constant. However, both V_{max} and K_m values were observed to be lower when

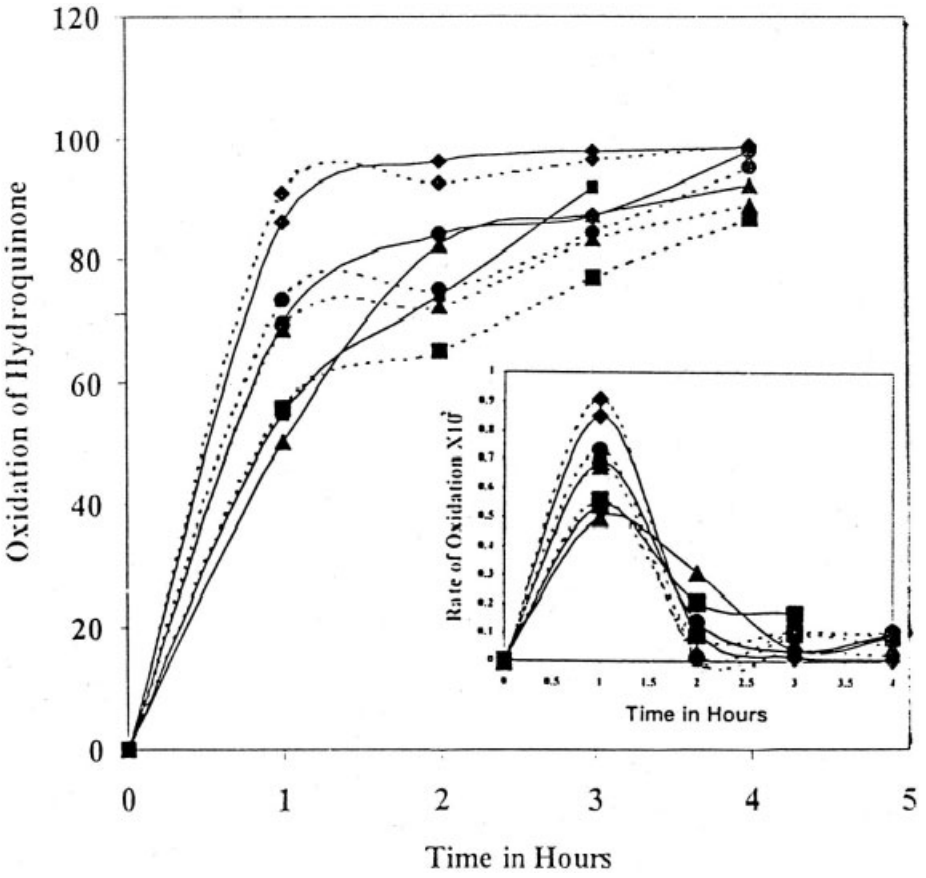

Figure 8 Effect of enzyme concentration on the rate of oxidation of hydroquinone at hydroquinone concentration 2 m*M;* free enzyme (—) and entrapped enzyme (—); 25 μg (■), 50 μg (▲), 75 μg (●), 100 μg (◆) enzyme.

the kinetics were controlled by concentration of substrate diffusion at a fixed enzyme concentration condition.

Storage studies

The storage of free and entrapped enzyme studied at 35°C (Figure 6) shows that the free enzyme loses about 50% activity in one week while the immobilized enzyme shows good storage stability and retains 50% of its activity for one month.

Oxidation of hydroquinone

The oxidation of hydroquinone was carried out by using both free and immobilized enzymes. It was observed that the initial rate of oxidation of hydroquinone by immobilized enzyme was lower than by free enzyme. But after 1 h, it showed reverse order. This could be due to the diffusional limitation of the substrate in the beads, which can be seen in the Figure 7. Effect of enzyme concentration on oxidation of hydroquinone was studied by using 25, 50, 75, and 100 μg of free and immobilized enzyme. The results are illustrated in Figure 8. The rate of oxidation increases with increase in enzyme concentration for both free and entrapped enzymes. It was observed that use of 100 μg of enzyme resulted in 90% of oxidation of hydroquinone in 1 h. The oxidation was also carried out at different pHs and it was found that in case of free enzyme the optimum pH for the oxidation is 6 whereas for entrapped enzyme it was 7 (Fig. 9). Hence the enzymatic reaction for the preparation of *p*-benzoquinone can be carried out at pH 7 and at room temperature, whereas the chemical process needs an acidic medium and less than 10°C temperature as the reaction is exothermic.

Diffusion coefficient

The diffusion coefficient (D_e) was calculated as discussed by Crank[24] by using eq. (2):

$$\frac{C(t)}{C(0)} = \frac{\alpha}{1+\alpha} + \sum_{n=1}^{\infty} \frac{6e^{-D_e q_n t/R}}{9/\alpha + 9 + q_n^2 \alpha} \qquad (2)$$

where t is the time at which the diffusion attains maximum, $C(t)$ is the concentration at time t, $C(0)$ is

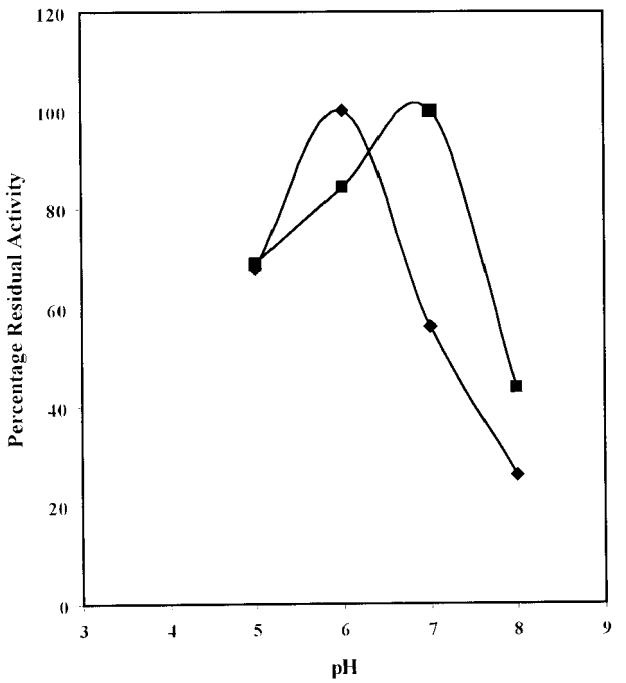

Figure 9 Effect of pH on the rate of oxidation of hydroquinone at 35°C for 4 h; free enzyme (◆) and entrapped HRP (■).

the original solute concentration, α is the ratio of the volume of solution and the beads, q_n is the the positive nonzero roots of the transcendental equation, and D_e is the effective diffusion coefficient.

Volume ratio of solution to the beads (α) is calculated from eq. (3):

$$\frac{M_t}{VC_0} = \frac{1}{1+\alpha} \tag{3}$$

where M_t is the amount of solute in the beads at time t, V is volume of solution, and C_0 is initial concentration of solute in the solution.

From the value of α partition coefficient, K_p can be calculated as follows:

$$K_p = \frac{3V}{4\pi R^3 n_b \alpha} \tag{4}$$

where V is the volume of the solution, R is the radius of the beads, n_b is the number of beads, and α is volume ratio of solution to the beads.

The concentration of the solute in the beads $C_b(t)$ was calculated from K_p and $C_s(t)$ using following equation where $C_s(t)$ is concentration of solute in the solution and $C_b(t)$ is the concentration of solute in beads at time t.

$$K_p = \frac{C_b(t)}{C_s(t)} \tag{5}$$

$$\tan q_n = \frac{3q_n}{3+\alpha q_n^2} \tag{6}$$

were taken from Table 6.1 given by Crank[24] for the values of α used in our work. By substituting these values in eq. (2), the effective diffusion coefficient (D_e) calculated for hydroquinone as substrate for K-carrageenan gel beads was found to be 0.27×10^{-10} and the partition coefficient was found to be 2.45. As the number of beads increase, the uptake of the solution from a fixed volume will increase. In our case we have taken a fixed amount and carried out the diffusion experiment, and the solute partitioning was found to be high. We have observed that the effective diffusion coefficient and the partition coefficient depend mainly on the number of beads and the concentration of solution. This method of finding the effective diffusion coefficient is simple and is easy to perform as we have taken the volume uptake and found the partition coefficient and effective diffusion coefficient. This system of ours can be easily applied to any substrate solution in which the solution as a whole goes into the beads. Thus it makes it applicable for finding K_p and D_e in similar cases.

CONCLUSION

HRP was immobilized on K-Carrageenan bead by entrapment technique. The immobilized enzyme found to be superior to the free enzyme under all conditions tested. The oxidation of hydroquinone was checked by using free and entrapped HRP, and it was found that about 90% oxidation was achieved in 1 h. The effective diffusion coefficient was calculated and found to be 0.27×10^{-10} m^2/s.

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