Immobilization of Horseradish Peroxidase on Chitosan for Use in Nonaqueous Media

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ABSTRACT: Chitosan, a natural polysaccharide, was used for the covalent immobilization of horseradish peroxidase, an enzyme of high synthetic utility, with the carbodiimide method. Of the enzyme, 62% was immobilized on chitosan when 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) was used as the peptide coupling agent. The influence of different parameters, such as the enzyme concentration, carbodiimide concentration, and incubation period, on the activity retention of the immobilized enzyme was investigated. Kinetic studies using horseradish peroxidase immobilized on chitosan revealed the effects of several parameters, such as the substrate hydrophilicity and hydrophobicity, the solubility of substrates in the medium, the solvent hydrophobicity, and the support aquaphilicity, on the catalytic activity of the immobilized enzyme in nonaqueous media. General rules for the optimization of solvents for nonaqueous enzymology based on the partitioning of the solvent were not applicable for the immobilized horseradish peroxidase. The catalytic efficiency was greatest when *o*-phenylene diamine was used as the substrate and least when guaiacol was used. The aquaphilicity of the support played an important role in the kinetics of the immobilized horseradish peroxidase in water-miscible solvents. The results were promising for the future development of chitosan-immobilized enzymes for use in organic media. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 1456–1464, 2003

Key words: enzymes; catalysis; supports

INTRODUCTION

Nonaqueous enzymology has emerged as an exciting field of research that provides a convenient and versatile tool for the synthetic organic chemist. However, the high concentration of an organic solvent in a reaction medium can lead to the inactivation of enzymes because of reversible changes in the protein structure, and prolonged incubation causes the irreversible inactivation of enzymes.[1,2] The high synthetic potential of these biocatalysts has necessitated the development of various methodologies to delay both reversible and irreversible inactivation. Several strategies have been devised for enhancing the stability and activity of enzymes in organic media, such as chemical modification,[3] immobilization,[4,5] molecular imprinting with substrates,[6] the addition of effectors,[7] and the use of surfactants.[8,9] Substantial attention has been devoted to the covalent immobilization of enzymes to porous, insoluble supports such as glass,[10] alumina,[11] silica,[11] and chitosan[12–21] because they possess a high catalytic activity per unit volume of the catalyst and minimum diffusion limitations and facilitate the diffusion of large substrate molecules into the porous structure of the matrix within which enzymes are bound.

Chitosan is a natural, hydrophilic polysaccharide made up of 2-amino-2-deoxy-D-glucose units linked by β-1,4-linkages, and it is obtained by deacetylation with a drastic alkaline treatment of chitin, which is the principal component of the exoskeleton of crustaceans and insects and some fungal cell walls. Chitosan has gained importance as an immobilization matrix in recent years because of its low cost and robust nature. There have been reports on the immobilization of several enzymes such as urease,[12] lipase,[13] β-galactosidase,[14] cellobiase,[15] tyrosinase,[16] invertase,[17] β-amylase,[18] alcohol dehydrogenase,[19] L-rhamnopyranosidase,[20] and horseradish peroxidase (HRP)[21] on chitosan. However, studies on the use of immobilized enzymes for biocatalysis in nonaqueous media are still scarce and have dealt with the properties of the immobilized enzyme in aqueous media only. Therefore, it is worthwhile to study the behavior of chitosan-immobilized peroxidase in organic solvents and to compare the kinetic parameters in different types of nonaqueous media with those observed in aqueous media.

HRP (Enzyme Commission Number: 1.11.17) belongs to the class of oxidoreductases that catalyze the oxidation reactions of various phenols and amines in the presence of H_2O_2. HRP has acquired considerable interest in the field of organic synthesis in recent

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years[22] because the enzyme catalyzes commercially important reactions such as phenol,[23,24] aniline polymerizations,[25,26] and the synthesis of specialty chemicals, including 3,4 dihydroxyphenylalanine (DOPA)[27] and bisphenol.[28] The majority of the work on HRP immobilization has focused on the use of immobilized enzyme preparations as biosensors.[29–31] The specific objectives of this study were to immobilize HRP on chitosan and to study the kinetics of the immobilize enzyme in different solvent systems for three different substrates. We chose HRP as the model enzyme for our study because of its high synthetic utility in nonaqueous media. In this article, we present a different method for the immobilization of HRP on chitosan that makes use of 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) (EDC) as the peptide coupling agent to form bonds between the amino groups of chitosan and the carboxyl groups of HRP. This method of immobilization eliminates the disadvantages associated with the use of the glutaraldehyde method,[32] which requires extensive washing procedures to remove the excess reagent. The activity of the immobilized enzyme was found to depend on various parameters, such as the EDC concentration, enzyme loading, and reaction time, and so these conditions were optimized to produce an immobilized enzyme preparation with maximum activity and stability in organic solvents.

EXPERIMENTAL

Materials

HRP type II (specific activity = 200 units/mg), EDC, and 2,2′-azino-bis-(3-ethyl benz-thiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma (St. Louis, MO); H_2O_2 was acquired from S.D. Fine Chemicals Ltd. (Mumbai, India). Chitosan was supplied by CIFT (Cochin, India). Guaiacol and orthophenylene diamine were obtained from S.D. Fine. All the salts used were analytical-grade, and the solvents were of the highest quality commercially available.

Preparation of the support

Chitosan was subjected to a drastic alkali treatment with 40% NaOH to ensure complete deacetylation. Chitosan (10 g) was heated at 90°C with four times its weight of 40% NaOH. It was filtered after 2 h and washed successively with distilled water, ethanol, and ether.

Immobilization of HRP

HRP (2 mg) was dissolved in 1 mL of distilled water. EDC (0.049 g) and 10 mg of chitosan were added, and the mixture was stirred slowly for 48 h. After 48 h, the immobilized enzyme preparation was filtered from the reaction mixture and washed successively with distilled water, a 0.1*M* acetate buffer (pH 6.3), 0.5*M* NaCl, and the acetate buffer again.

The activity of the immobilized enzyme was calculated with the following formula:

$$\text{Activity retention (\%)} = \frac{\text{Immobilized enzyme activity}}{\text{Initial activity of the enzyme solution}} \times 100$$

After being washed, the chitosan-immobilized enzyme was suspended in a 0.05*M* acetate buffer (pH 6.3) and stored in a refrigerator until it was used. Just before the experiments were performed, the stored immobilized enzyme was dried over a Buchner funnel for a final moisture content of 0.5%/g of moist chitosan flakes.

Protein estimation

The protein concentration was determined with Lowry et al.'s[33] method with the Folin reagent, and the absorbance was read at 660 nm with a spectrophotometer. The protein used to establish the standard curve was the same HRP that was used for the immobilization. The protein estimation of the immobilized enzyme was performed according to the procedure described by Burgess et al.[46] with a slight modification. A weighed amount of the immobilized enzyme was suspended in a known quantity of distilled water, the phenol reagent was added, and the tubes were shaken at 120 strokes per minute for 30 min. After centrifugation, the supernatant absorbance was measured at 660 nm.

Assay of peroxidase

The HRP activity was assayed with the ABTS method.[34] The assay mixture consisted of 0.6 m*M* ABTS and 1.2 m*M* H_2O_2 in a 67 m*M* phosphate buffer (pH 6.0). The activity was calculated from the increase in the absorbance per minute at 420 nm. The activity of the immobilized HRP was determined with the procedure for immobilized enzymes.[35] The enzyme matrix was kept outside the light path with stirring, aliquots of the reaction mixture were introduced into the cuvette at definite intervals of time, and the increase in the absorbance per minute was monitored.

Effect of the EDC concentration on the activity of the immobilized enzyme

To study the effect of the EDC concentration on activity retention, we used EDC concentrations ranging from 20 to 250 m*M* for immobilizing HRP to chitosan.

Effect of the enzyme loading on the activity

The effect of the enzyme loading on the activity of the immobilized enzyme preparation was studied by the performance of immobilization with various amounts of HRP (0.5–7.5 mg).

Effect of the incubation time on the activity

The immobilization of HRP was carried out for different period of times ranging from 15 to 96 h. The activity of the immobilized enzyme was measured, and the activity retention was calculated.

Effect of the organic solvent on the activity of chitosan-immobilized HRP

The oxidative coupling reaction of *o*-phenylene diamine (OPD) to 2,3-diamino phenazine (DAP) was chosen as the model reaction for investigating the effect of organic solvents on the immobilized enzyme activity. The initial reaction rate of the oxidation reaction in different organic solvents was calculated from the increase in the absorbance at 450 nm caused by the product formed. Solutions of OPD in solvents of various log *P* values were prepared (*P* is the octanol/water partition coefficient of the solvent), and the reaction was carried out with 5 m*M* OPD, 0.5 m*M* H_2O_2, and 10 mg of the immobilized enzyme.

Kinetics of the immobilized enzyme

Two reactions, the oxidative coupling reaction of OPD to DAP and the oxidation of guaiacol to dimethoxybiphenoquinone, were selected for the kinetic studies of chitosan-immobilized HRP. The kinetic studies were performed in three different solvent systems: an aqueous system, an aqueous–organic mixture (80% dioxane), and a microaqueous system (toluene).

Oxidation of OPD to DAP

Solutions of OPD (1%) were prepared in 80% dioxane, toluene, and water. The reaction mixture consisted of immobilized HRP (10 mg in nonaqueous media and 2 mg in aqueous media), 0.5 m*M* H_2O_2, and various concentrations of OPD. The reaction rate was calculated by the monitoring of the increase in the absorbance at 450 nm due to the formation of DAP.

Oxidation of guaiacol to dimethoxybiphenoquinone

Guaiacol solutions (30 m*M*) were prepared in water, toluene, and 80% dioxane. The reactions were carried out in the respective solvents with immobilized HRP (20 mg for nonaqueous media and 4 mg for aqueous media), guaiacol of various concentrations, and 4 m*M* H_2O_2. Several kinetic runs were performed with various guaiacol concentrations, and the kinetics of the reaction were followed by the monitoring of the increase in the absorbance of the colored product at 470 nm. Kinetic constants were determined from straight-line plots (Lineweaver–Burk plots) of the Michaelis–Menten equation.

RESULTS AND DISCUSSION

The use of the carbodiimide activation of carboxyl groups has been a common technique for protein immobilization. The maximum activation of carboxyl groups by EDC has been reported to be at pH 4. However, because the reagent is more stable at neutral pH and because of the high conjugation yields reported for HRP to soluble polysaccharide matrices such as diethyl aminoethyl (DEAE) and carboxymethyl cellulose (CMC) in distilled water with dicyclohexyl carbonate (DCC),[36] the immobilization was accomplished in distilled water.

Effect of the carbodiimide concentration

Th effect of the EDC concentration on the activity of the crosslinked enzyme is illustrated in Figure 1. With an increase in the EDC concentration, the amount of enzyme covalently attached to the matrix increased up to 200 m*M*. At very low concentrations of EDC, a smaller number of bonds formed between the enzyme and the support. As the concentration of EDC increased, more enzyme molecules were covalently bound to the support through the formation of peptide bonds between the amino groups of chitosan and the carboxyl groups of HRP.

When the concentration was increased further, a decrease in the activity of the immobilized enzyme was observed that might be due to the nonspecific activation of the amino groups of the enzyme by the carbodiimide when present in excess.

Effect of the enzyme loading

The variations in the amount of enzyme added to the support altered the activity of the immobilized enzyme preparation, as evident from Figure 2. The activity of the immobilized enzyme increased with an increase in the enzyme loading up to 2.5 mg. A further increase in the enzyme loading resulted in decreased activity. The drop in the enzyme activity with increasing substitution on the surface might have been due to the increase in substrate diffusional limitations.

Effect of the incubation period on the activity

As evident from Table I, an optimum incubation period of 48 h was required for the maximum immobi-

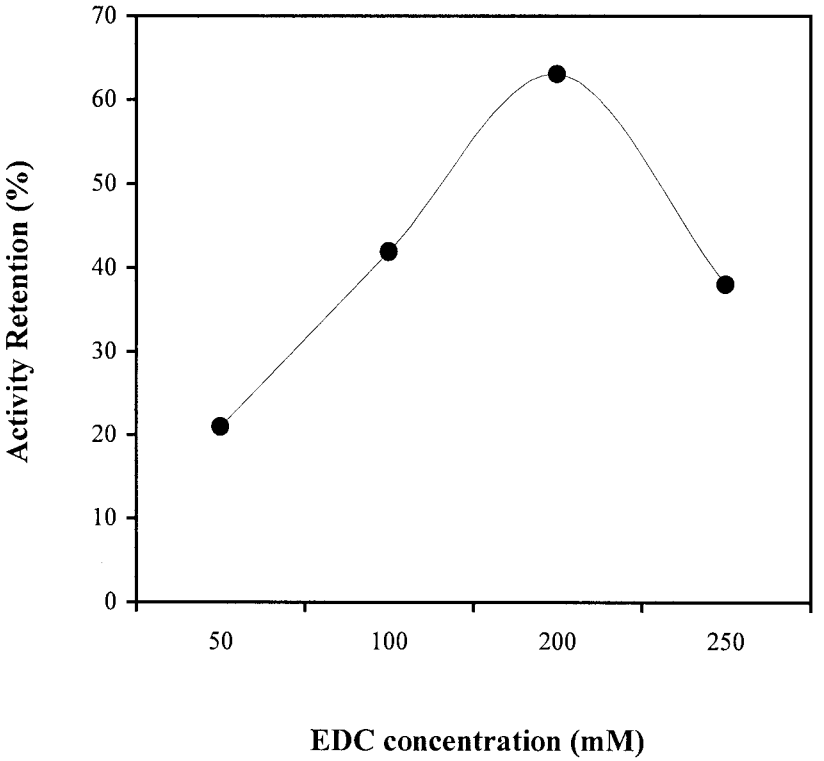

Figure 1 Effect of the EDC concentration on the activity of immobilized HRP. HRP (2 mg) was dissolved in 1 mL of distilled water. Chitosan (10 mg) was added, and the immobilization was performed with different concentrations of EDC (50–250 m*M*). The immobilization was allowed to proceed for 48 h at 4°C. The immobilized enzyme was washed successively with distilled water, a 0.1*M* acetate buffer (pH 6.3), 0.5*M* NaCl, and the acetate buffer again.

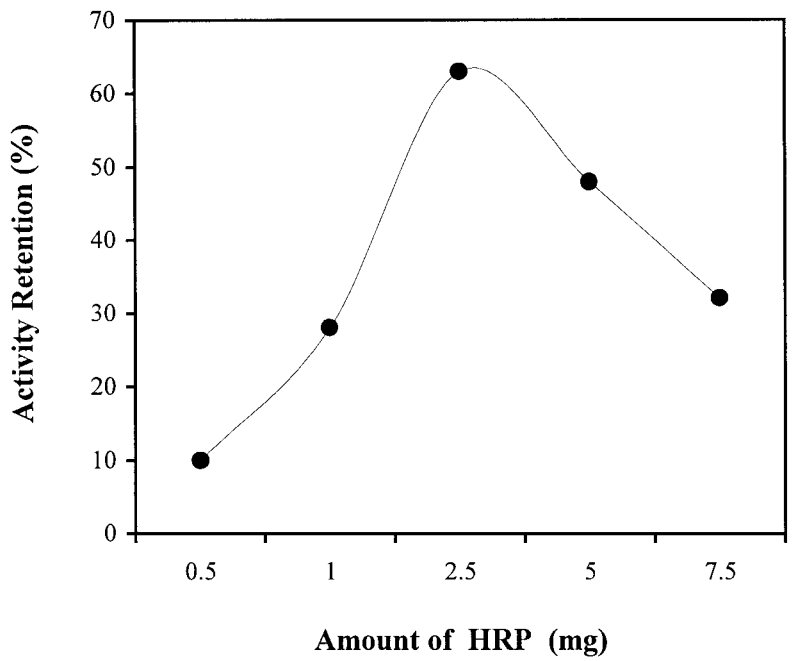

Figure 2 Effect of the enzyme loading on the immobilization of HRP on chitosan. Various amounts of HRP (0.5, 1, 2.5, 5, and 7.5 mg) were dissolved in 1 mL of distilled water. Chitosan (10 mg) was added, and the immobilization was performed with 200 m*M* EDC. The immobilization was allowed to proceed for 48 h at 4°C. The immobilized enzyme was washed successively with distilled water, a 0.1*M* acetate buffer (pH 6.3), 0.5*M* NaCl, and the acetate buffer again.

TABLE I
Effect of the Incubation Period on the Activity Retention of HRP

Incubation time (h)	Activity retention (%)
16	18
24	41
48	67
96	30

HRP (2 mg) was dissolved in 1 mL of distilled water. Chitosan (10 mg) was added, and the immobilization was carried out with 200 m*M* EDC. The system was incubated at 4°C for different amounts of time (16, 24, 48, and 56 h). The immobilized enzyme was washed successively with distilled water, 0.1*M* acetate buffer (pH 6.3), 0.5*M* NaCl, and again with acetate buffer.

lization of HRP on chitosan. Beyond this limit, the activity of the immobilized enzyme decreased, possibly because of the denaturing effect of EDC when it was incubated for a longer period.

Activity of chitosan-immobilized HRP in organic solvents

The activity of the immobilized enzyme in different organic solvents is shown in Figure 3. Among the different solvents investigated, the maximum catalytic activity was observed in toluene, which was followed by chloroform and ethyl acetate. This observation is similar to that reported by Kazandjian et al.[37] for the peroxidase (deposited on glass powder) -catalyzed oxidation of *p*-anisidine in organic solvents. The influence of the matrix on the solvent tolerance varied with the solvent used but did not correlate with a solvent parameter such as log *P*. The behavior of chitosan-immobilized HRP might have been due to the differences in the water stripping capacities of the solvents from the enzyme. The solvents used for this study belong to three groups based on their affinity for water. Acetone, dioxane, and the protic solvents (ethanol, methanol, and isopropanol) are extremely hydrophilic, infinitely water-miscible solvents that strip off the water bound to the enzyme essential for the catalytic activity.[38] Toluene and chloroform belong to a group of very hydrophobic, water-immiscible solvents that do not strip the essential water away from the enzyme and, therefore, support enzyme activity. Ethyl acetate belongs to a separate group of solvents that are less hydrophobic and still not completely miscible with water. This group of solvents removes water from the enzyme if not presaturated with water.

Protic solvents were least efficient in supporting catalysis, and the catalytic activity of the immobilized enzyme increased with an increase in the hydropho-

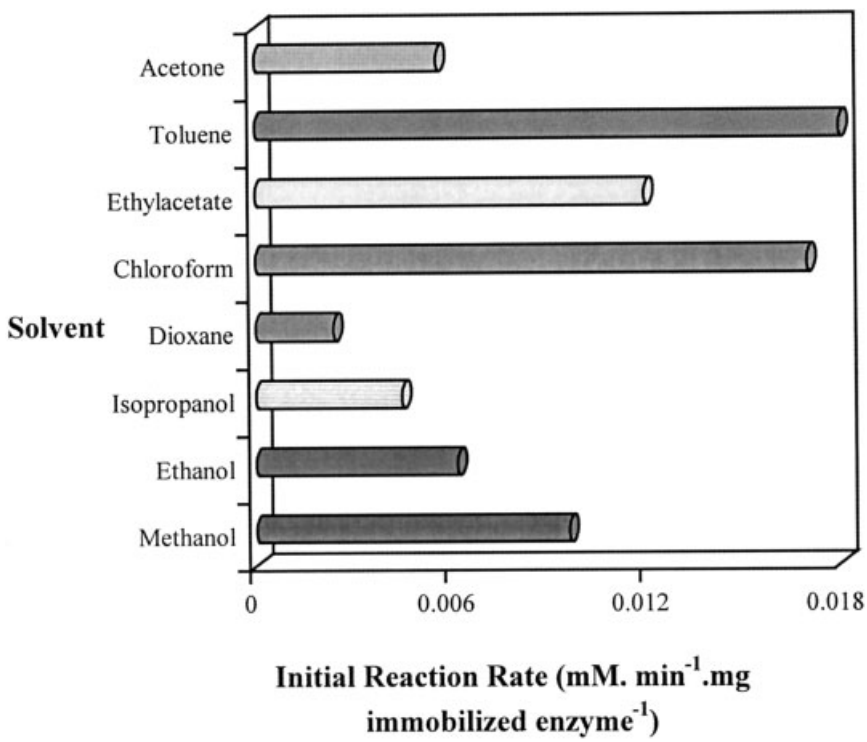

Figure 3 Effect of organic solvents on the activity of HRP immobilized on chitosan. Solutions of OPD (1%) in solvents with various log *P* values were prepared, and the reaction was performed with 5 m*M* OPD, 0.5 m*M* H_2O_2, and 10 mg of the immobilized enzyme. The reaction rate was calculated by the spectrophotometric monitoring of the increase in the absorbance at 450 nm.

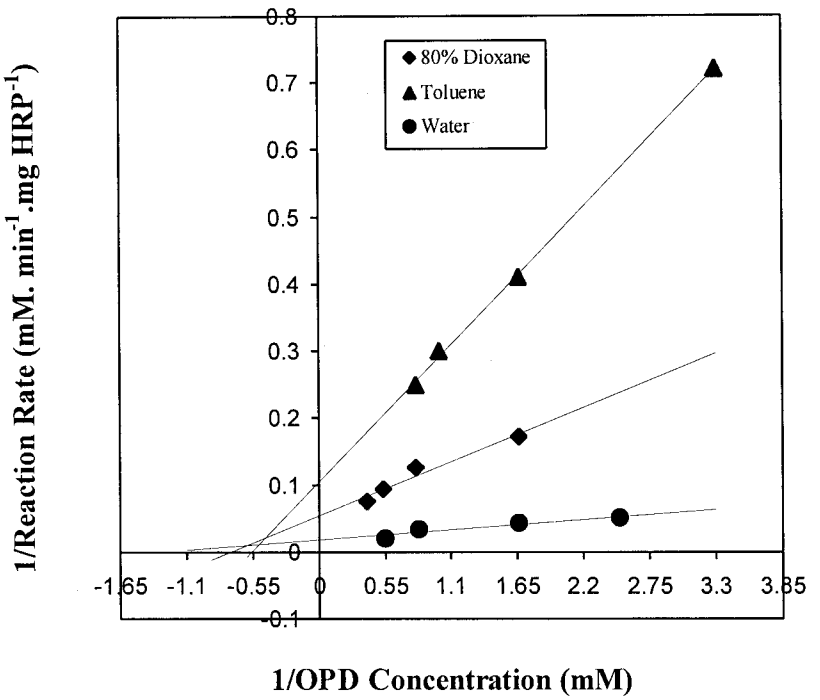

Figure 4 Lineweaver–Burk plots for chitosan-immobilized HRP in different solvent systems for the OPD–DAP reaction. Solutions of OPD (1%) were prepared in 80% dioxane, toluene, and water. The reaction mixture consisted of immobilized HRP (10 mg in the nonaqueous medium and 2 mg in the aqueous medium), 0.5 m*M* H_2O_2, and OPD of various concentrations. The reaction rate was calculated by the monitoring of the increase in the absorbance at 450 nm due to the formation of DAP.

bicity of the protic solvent. This might have been due to the preferential exclusion of more hydrophobic solvents from the hydrophilic microenvironment of chitosan. A comparable observation was reported by Pliura and Jones[39] for chymotrypsin immobilized on Sephadex.

Kinetic studies of immobilized HRP in different solvent systems

In this work, we studied the kinetics of immobilized HRP in three different solvent systems: an aqueous system, a completely miscible aqueous–organic mixture, and a water-immiscible organic solvent (Figs. 4 and 5). The kinetic parameters for immobilized HRP and native HRP for different solvent–substrate systems are summarized in Table II (K_{cat} is the catalytic efficiency and is defined as V_{max}/K_m). It was obvious that the immobilized enzyme exhibited the highest catalytic efficiency and substrate specificity in 80% dioxane for OPD in all the solvent systems used for the study. The catalytic efficiency of immobilized HRP was lowest for guaiacol in toluene, and the corresponding substrate specificity was also low. Chitosan-immobilized HRP showed an increase in the Michaelis constant K_m in both solvents for all the substrates in comparison with water, but the V_{max} value showed a different trend (V_{max} is the maximum reaction rate). For OPD, V_{max} was high in the water-miscible solvent dioxane, whereas for guaiacol, V_{max} had a high value in toluene in comparison with that in dioxane. The differences in the reaction rates in various solvents might be explained by differences in the partitioning of the substrates or products between the bulk solvent phase and the biocatalyst phase. Blanco et al.[40] reported a similar observation for immobilized chymotrypsin in water-immiscible solvents.

It is obvious from Table II that all the kinetic parameters except K_m decreased with a decrease in the polarity of the solvent for both substrates used in the study. Immobilized HRP exhibited an increase in K_m values and a corresponding decrease in V_{max} values as the water content of the solvent was reduced. A similar observation was reported by Akkara et al.[41] for the HRP-catalyzed oxidation of *p*-cresol. It was clear from their study that the increase in K_m with the ethanol content might be due to the diminishing affinity of the enzyme toward substrate binding. A decrease in the reaction rates could not be due to enzyme denaturation caused by the solvent but rather was due to the strong effect of the solvent on the partitioning of the substrate to the enzyme active site.[41]

The substrate specificity (V_{max}/K_m) of the immobilized enzyme, which provided a measure of the relative specificity of the enzyme, was maximum for orthophenylene diamine and very low for guaiacol; this indicates the poor binding capacity of the substrate

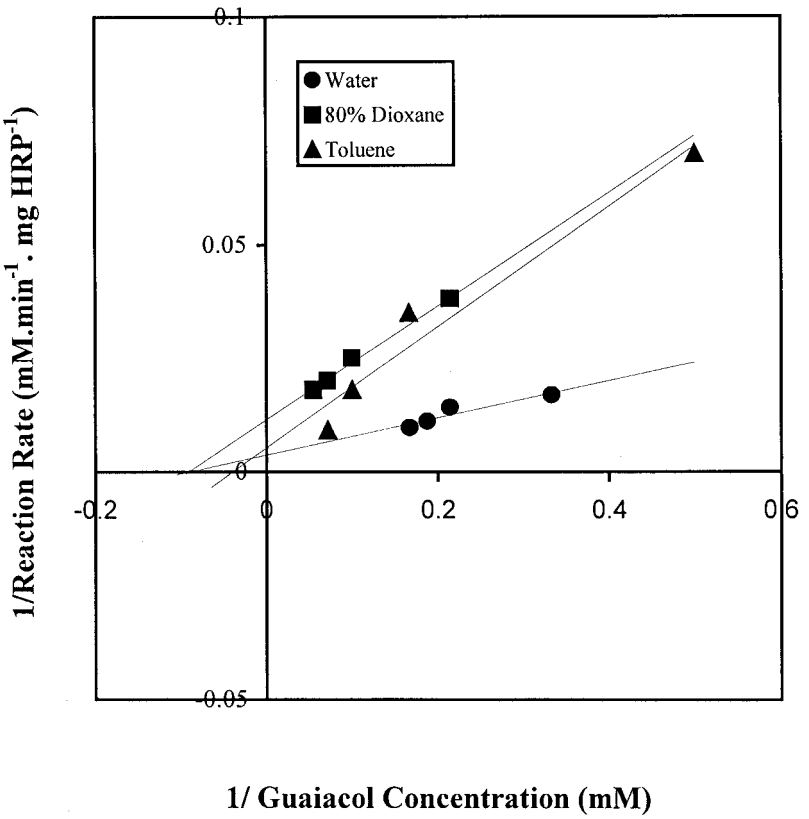

Figure 5 Lineweaver–Burk plots for chitosan-immobilized HRP in different solvent systems for the oxidation of guaiacol. Guaiacol solutions (30 m*M*) were prepared in water, toluene, and 80% dioxane. The reactions were performed in the solvents with 10 mg of the immobilized enzyme, 4 m*M* H_2O_2, and guaiacol of various concentrations. The kinetics of the reaction were followed by the monitoring of the increase in the absorbance of the colored product at 470 nm.

molecule to the enzyme. It was evident from the study that dioxane exerted a significant effect on the substrate specificity of immobilized peroxidase. A similar phenomenon was reported by Ryu and Dordick[42] in their study on the kinetic behavior and substrate specificity of HRP in water-miscible solvents.

TABLE II
Comparison of the Kinetic Parameters of Chitosan-Immobilized HRP with Native HRP for Different Substrate–Solvent Systems

Form of enzyme	Substrate	Solvent	K_m (m*M*)	V_{max} (mM^{-1}min^{-1} mg of enzyme)	V_{max}/K_m	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
	Guaiacol	Water	11.11	270	24.3	648	58.3
Chitosan-HRP	Guaiacol	80% dioxane	22.72	200	8.8	160	7.04
	Guaiacol	Toluene	25	133	5.32	106.4	4.26
	OPD	Water	0.9	40	44.44	480	533
	OPD	80% dioxane	1.43	20	14	120	84
	OPD	Toluene	1.82	6.45	3.54	21.24	11.67
Free HRP	OPD	Water	0.71	111.11	156.5	533.3	751
	OPD	80% dioxane	1.11	71.4	64.35	342.7	308.7
	OPD	Toluene	2.85	41.67	14.62	200	70.18
	Guaiacol	Water	3.33	125	37.5	600	180
	Guaiacol	80% dioxane	4	100	25	480	120
	Guaiacol	Toluene	8.33	62.5	7.5	300	36

The reaction was performed in the respective solvent with 0.5 m*M* H_2O_2, immobilized HRP (5 mg in organic media, 2 mg in aqueous medium) and various concentrations of OPD. The reaction rate was calculated by monitoring the increase in absorbance at 450 nm due to the formation of DAP. Kinetics of the guaiacol oxidation were studied with immobilized HRP (20 mg for nonaqueous media and 4 mg for aqueous media), 4 m*M* H_2O_2, and various guaiacol concentrations. The increase in absorbance of the product at 470 nm was monitored.

The ability of the enzyme to use the free energy of binding with the substrate determines its substrate specificity and catalytic efficiency, and this binding energy reflects the difference between the binding energies of the substrate–enzyme and substrate–solvent interactions. Therefore, the kinetic parameters describing enzyme function, such as V_{max}, K_m, and the catalytic specificity (V_{max}/K_m), depend strongly on the nature of the solvent. Shifting the reaction medium from water to an organic medium was accompanied by consequential changes in the observed kinetics of the enzyme.

It has been reported in the literature that peroxidase catalysis in nonaqueous media is strongly affected by both the substrate and solvent hydrophobicities. Ryu and Dordick[42] studied the peroxidase-catalyzed oxidation of phenols in organic solvents as a model to elucidate the solvent-induced kinetic alterations of enzymatic catalysis, particularly with respect to substrate and solvent hydrophobicities. Their study unveiled the existence of a linear free energy relationship between the catalytic efficiency and substrate and solvent hydrophobicities. The same group investigated the effect of water-miscible organic solvents on the intrinsic kinetics and substrate specificity of HRP immobilized on glass beads.

Concerning the effects of the different solvent systems on different substrates, some important conclusions could be derived. First, the presence of less polar solvents exerts a more deleterious effect on the enzyme activity than the presence of more polar solvents. This behavior is clearly opposite to that reported in the literature for the free enzyme. Blanco et al.[40] reported similar behavior for immobilized chymotrypsin in nonaqueous solvents.

Possible reasons for the inactivation of biocatalysts in nonpolar solvents suggested by Halling[43] include

1. The binding of the solvent to the enzyme.
2. The alteration of the interaction between water molecules and other polar species in the environment that weakens the hydrophobic effect, which plays an important role in stabilizing the native structure of the enzyme.

Another factor contributing to the activity of the enzyme in different solvent systems is the solubility of the substrate in the solvent. For a microaqueous system, in which contact of the biocatalyst with the bulk organic phase is involved, log P is not the parameter controlling enzyme activity, and the bulk solvation properties are appropriate in describing the kinetic behavior of biocatalysts in such media. Kinetic constants of the immobilized enzyme were different for different solvent systems, reflecting their different solvation or partition behaviors. Some of the observed behavior at low water contents (microaqueous systems) must involve molecular effects on the enzyme and its complexes.

Second, the nature of the support material influences the kinetics of the immobilized enzyme in the case of a water-miscible organic solvent. A solvent–aqueous mixture containing the substrate extends to the surface of the biocatalyst because of the hydrophilic nature of the support, and this results in high catalytic turnovers. This competition of the support material for water and the resulting effects of the activity of the immobilized enzyme preparation were demonstrated by Reslow et al.[44] A similar observation was reported by Khmelnitsky et al.[45] for immobilized amine oxidase in nonaqueous media. According to Khmelnitsky et al., for reaction systems based on immobilized enzymes dispersed in organic solvents, part of the available water is dissolved in the organic solvent, and the remaining water is bound to the support and the enzyme and usually does not form a visually detectable separate phase. When the overall concentration of water in the system is high, it may form a continuous aqueous microphase within pores of the solid support, actually resulting in a biphasic system with a supported aqueous phase. In this case, the immobilized enzyme experiences essentially an aqueous microenvironment and may exhibit catalytic properties similar to those observed in bulk aqueous solutions. When the concentration of water is low, the amount of available water may not be sufficient to form the aqueous microphase, and this leads to significant changes in the hydration state of the immobilized enzyme, which strongly affects the catalytic activity.

CONCLUSIONS

The carbodiimide-mediated covalent immobilization of HRP on chitosan was found to be an effective method for the preparation of biocatalysts stable in nonaqueous media. Conventional rules for the optimization of solvents for nonaqueous enzymology based on their log P values are not applicable to the immobilized enzyme. It is evident from this study that the immobilization of enzymes results in conformational changes that result in changes in the substrate specificity of enzymes. Evidently, the behavior of immobilized enzymes in different solvent systems is a complex one and involves several parameters, such as the activity and stability of the enzyme derivative, the solubility and partitioning of the substrates and products, and the aquaphilicity and aquaphobicity of the support used for immobilization.

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