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Effect of organic solvents on peroxidases from rice and horseradish: Prospects for enzyme based applications

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ABSTRACT

A feasibility test for rice peroxidase (RP) enzyme as a substitute for horseradish peroxidase (HRP) was carried out. The activity of HRP was maximum at 30 °C with pH 6.0–7.0. The purified rice peroxidase showed optimum activity at 30 °C with pH 7–8 and was thermostable till 68 °C, which is higher than the temperature reported for HRP. RP obeyed Michaelis–Menten kinetics. With increasing substrate concentrations, RP and HRP had V_{max} as 8.23 μM min^{-1} and 4.21 μM min^{-1} and K_m as 5.585 and 3.662 mM, respectively. In 10% 1,4-dioxane and ethanol, RP exhibited 2 and 1.3 times higher activity, respectively than HRP. Shelf life studies show RP to be significantly stable till 60 h in 20% 1,4-dioxane and till 12 h in ethanol. The activity of RP/HRP increased gradually with 0%–40% ethanol or 0%–30% 1, 4-dioxane till 20 h with a sharp decline thereafter. The stability of HRP and RP reduced with increasing storage period. Enzyme efficiencies compared as V_m/K_m showed water miscible organic solvents, *viz.*1, 4-dioxane and ethanol, to exhibit a regular decrease in V_m/K_m with increase in organic solvent concentration whereas, a reverse trend was observed with water–immiscible solvent like chloroform. The relative activity of RP and HRP enzymes upon immobilization on poly-5-carboxy-indole shows increasing enzyme activity with time and with guaiacol/dopamine hydrochloride as substrates. Immobilized RP had a better relative activity with dopamine as substrate than immobilized HRP, whereas with guaiacol both RP and HRP had a comparable activity upon immobilization. Results suggest rice peroxidase to be a cheaper and convenient enzyme system for immobilization using organic solvents. The high thermal stability, more stability in organic solvents and longer shelf life of RP over the immobilizing matrix suggest conducting polyindole having carboxyl functional groups to be a suitable matrix for the covalent entrapment of rice peroxidase through amide linkage. Good sensitivity and fast response to dopamine makes RP a suitable enzyme system for monitoring dopamine levels in aqueous medium.

1. Introduction

Peroxidases [EC.1.11.1.7] catalyze the oxidation of wide range of organic and inorganic substrates in presence of H_2O_2 [1] as shown in the following equation:

$$H_2O_2 + \text{Peroxidase}_{red} \rightarrow \text{Peroxidase}_{ox} + H_2O$$

$$\text{Peroxidase}_{ox} + \text{Substrate}_{red} \rightarrow \text{Peroxidase}_{red} + \text{Substrate}_{ox} \quad (1)$$

Owing to this property they are receiving increased attention for potential use in biotechnological, clinical, industrial applications [2] and for other analytical purposes [3–5].

Biosensors based on enzyme-catalyzed reactions are being commercially explored nowadays for detection of various analytes in biological samples [6]. Availability of immobilization techniques employing various matrices have large scale utilization of biosensors owing to their ability to operate in a continuous way, requiring less amount of expensive enzyme, increased thermostability, operational stability, reusability and recovery [7].

Most of the peroxidase based biosensors employ commercially available horseradish peroxidase (HRP) and expensive matrices which makes them costly. Therefore, other peroxidases with enhanced stability, varied catalytic properties and increased activity are continuously sought for from microbes, animals and plants [7,8].

Functionalized conducting polymers are now being widely used for developing biosensors [9]. Since the proper immobilization and activity retainment plays a crucial role in developing any enzyme based reproducible application, testing of the enzyme activity in various organic solvents used during polymer synthesis and immobilization procedure is of considerable importance [10].

The present study includes feasibility and stability studies of low cost, widely available RP and commercially available but relatively expensive HRP in varying concentrations of different

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organic solvents and shelf life. Kinetics of RP are also carried out in organic solvents for their potential application in immobilization studies, a prerequisite for biosensor development.

Additionally, a comparative immobilization study is also performed with RP and HRP estimating dopamine levels in samples mimicking biological samples.

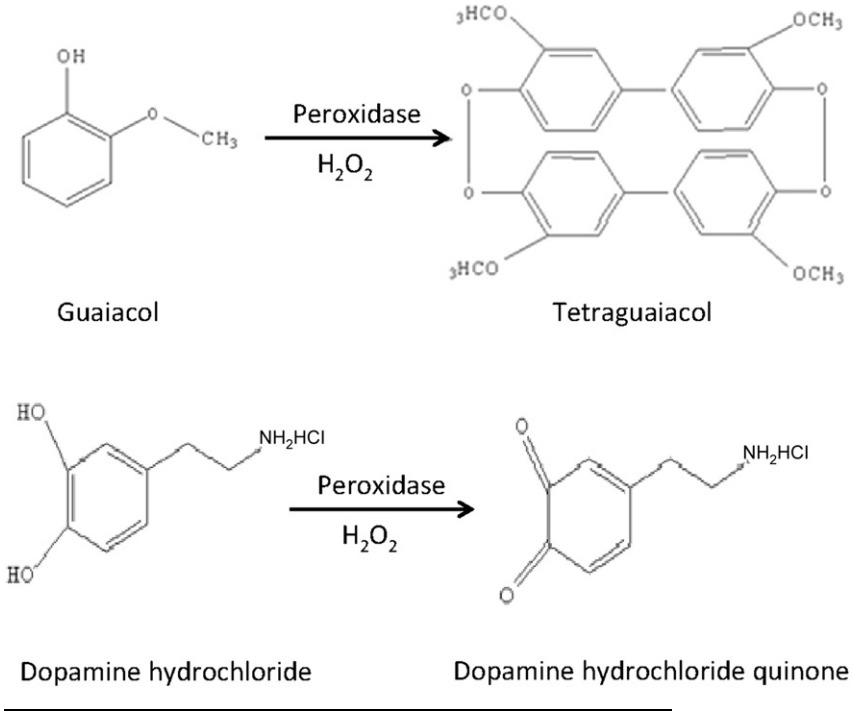
2. Experimental

2.1. Chemicals and reagents

BSA and HRP (SRL), guaiacol, H_2O_2, Folin-Ciocalteau phenol reagent (Loba-Chemie), poly 5-carboxy- indole (Sigma), Dopamine hydrochloride (Sigma) and all other chemicals and solvents used were of analytical grade (Hi-media or E.Merck).

2.2. Plant material

Rice seeds *cv*. Bh-1 were obtained from Indian Council of Agricultural Research, Barapani, Shillong, India.



(2)

2.3. Purification of RP from rice seedlings

Surface sterilized seeds of rice *cv.* Bh-1 were raised for 15 days in sand culture saturated with Hoagland's nutrient solution [11]. Seedlings were uprooted and used as shoot and root samples for enzyme extraction. Rice seedlings were homogenized in 50 mM sodium phosphate buffer, pH 7.0 using chilled mortar and pestle at 4 °C temperature. Homogenates were centrifuged at 15,000 rpm for 30 min [12] and supernatant obtained was subjected to 20%–90% ammonium sulfate precipitation on ice bath. Resulting solution was again centrifuged at 15,000 rpm for 30 min. Pellet obtained was resuspended in minimum volume of 20 mM HEPES, pH 7.0 containing 1 mM EGTA and 0.1% Tween-20. 2 mM $CaCl_2$ was added to the suspension for enzyme stability and subsequently applied to a calcium-polygalacturonate-polyacrylamide gel column (3.0 × 7.5 cm) [13,14]. The calcium-polygalacturonate-polyacrylamide gel column was then properly washed with HEPES, pH 7.0 containing 2 mM $CaCl_2$ and 0.1% Tween prior to elution. Bound protein (rice peroxidase) was then eluted using 0.5 M NaCl in 20 mM HEPES, pH 7.0. Peroxidase activity was measured by ELICO-SL-159 (India) UV–vis Spectrophotometer using 1.0 cm matched cells. The peroxidase active fractions were pooled, concentrated and desalted using centricon-10 (Amicon, Mllipore).

2.4. Rice peroxidase assay

The activity of RP was performed at each step of purification [15]. The reaction medium consisted of 3.9 ml 40 mM Na–P buffer (pH 6.1), 0.5 ml 9 mM guaiacol or 9 mM dopamine hydrochloride as an electron donor, 0.5 ml 2 mM H_2O_2 followed by addition of 0.1 ml of enzyme at 37 °C. The enzyme activity was monitored at 470 nm for guaiacol [15] and 490 nm for dopamine [16], at 30 s interval for 3 min. All protein estimations were carried out at 660 nm using Folin-Ciocalteau reagent [17] and BSA (SRL) as standard. Reaction of peroxidase with the two substrates using H_2O_2 has been shown in the following equation:

2.5. Catalytic stability of RP and HRP in organic solvents

Organic solvent profiles of RP and HRP samples were carried out at room temperature with exposure times of 1 h. The solvents used were tetrahydrofuran (THF), 1,4-dioxane, ethanol, dimethylsulfoxide (DMSO), cyclohexane, chloroform and dichloromethane. Reaction mixtures were set up with increasing percent volumes (10% (v/v) increments) of organic solvents in 40 mM Na–P buffer, pH 6.1 [18]. Hundred microliters were withdrawn from each reaction mixture and assayed under the standard conditions as stated above.

2.6. Effect of organic solvents on shelf life of RP and HRP

Stability of peroxidase in organic solvents is important for timely utilization and immobilization of peroxidase. The activity of RP and

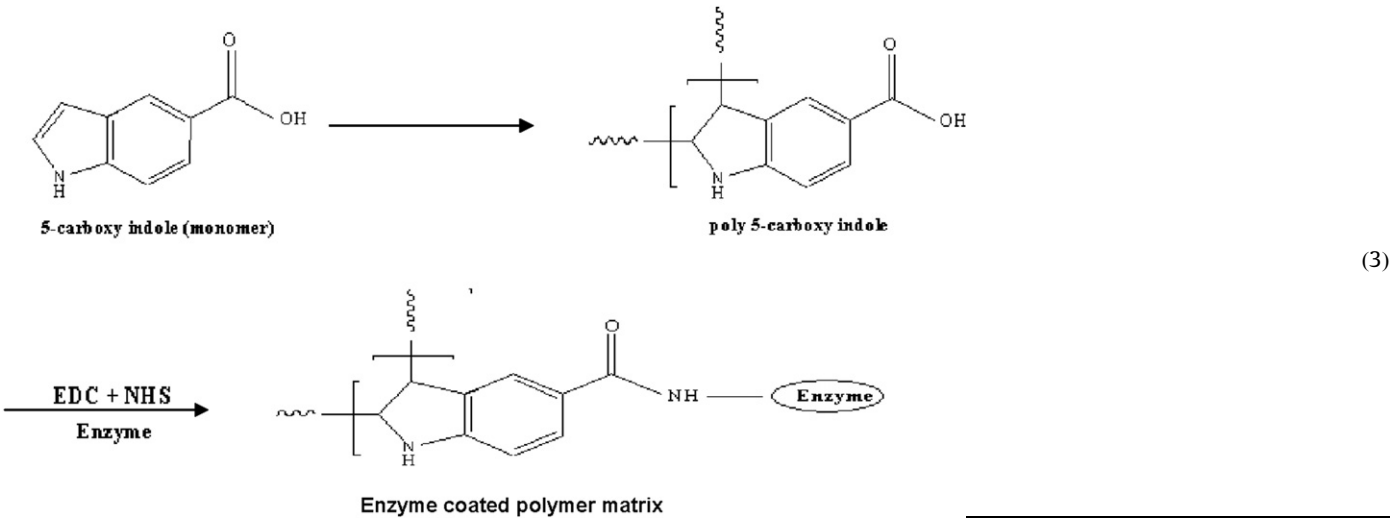
HRP was measured in solvents tetrahydrofuran (THF), 1,4-dioxane, ethanol and chloroform, by differing the percent of these solvents and storage periods (0, 12, 24 and 60 h) at room temperature. Reaction mixture as earlier contained 40 mM Na–P buffer (pH 6.1) and increasing volumes of organic solvents in 10% (v/v) increments, up to a maximum of 40%–60% with increasing storage time.

2.7. Kinetic studies of RP in organic solvents

The effect of varying substrate concentrations on RP in varying concentrations of organic solvents was studied. 2, 4, 6, 8 and 10 mM concentrations of guaiacol were prepared in 10% (v/v) increments of chloroform, ethanol and 1,4-dioxane. Double reciprocal plots were constructed against $1/V$ vs. $1/S$ and respective K_m values for each solvent were calculated.

2.8. Immobilization studies with RP and HRP

Immobilization of RP and HRP was accomplished on 5-carboxy indole polymer (functionalized conducting polymer) shown as reaction 2, to test the feasibility of RP for biosensing of biomolecule dopamine. For immobilization, small glass plates coated with poly-5-hydroxy indole polymer dissolved in THF and coupled with ethyl-dimethylaminopropyl carbodiimide (EDC) and N-hydroxy-succinimide (NHS) were incubated for 1.5 h in Tris–HCl (pH 7.0) [19]. Subsequently, 50 μl of RP or HRP was added on plates dropwise for adhering of enzyme to the immobilization matrix. Plates were dip cleaned in distilled water for removal of unbound enzyme and then freeze-dried at 4 °C. Following freeze-drying the polymer layer was scraped in 1.95 ml of 40 mM Na–P buffer (pH 6.1) to which 250 μl of 9 mM guaiacol or 9 mM dopamine was added and reaction was initiated with the addition of 250 μl of 2 mM H_2O_2. The activity of RP/HRP was monitored at 470 nm with guaiacol and at 490 nm with dopamine as substrate at 37 °C and at 30 s intervals for 3 min. The % relative activity of immobilized RP and HRP with the two substrates was calculated. A brief schematic representation of the immobilization procedure has been shown in the following equation:



(3)

3. Results and discussion

3.1. Activity of RP and HRP with guaiacol as substrate

The activity of HRP was maximum at 30 °C with pH 6.0–7.0 [14]. The purified rice peroxidase showed optimum activity at 30 °C with pH 7–8 and was thermostable till 68 °C [14], which is higher than the temperature reported for HRP [20]. The activity of HRP was higher than RP in aqueous medium which might be due to more stabilization of HRP by intramolecular disulfide bonds [21].

Effect of 2, 4, 6, 8 and 10 mM concentrations of substrate (guaiacol) on RP and HRP activity shown in Fig. 1, suggests the calculated value of V_{max} for RP to be 8.23 μM min^{-1} and for HRP to be 4.21 μM min^{-1}. K_m for RP and HRP was 5.585 and 3.662 mM respectively. Straight line of the Lineweaver Burk double reciprocal plot confirms the obedience of both RP and HRP for Michaelis–Menten equation (Fig. 1(A), (B), (C) and (D)).

3.2. Catalytic stability of RP and HRP in various organic solvents

Stability of peroxidase in organic solvents is of considerable importance for its broad range utilization. This study was performed for selection of an appropriate organic solvent that can be further used as a polymer dissolution agent for immobilization of RP for enzyme based applications. The percent relative activity of RP and HRP in varying concentrations of the organic solvents is shown in (Fig. 2(A)–(G)). The relative stability of RP in organic solvents (measured as the percent relative activity of the enzyme when compared to its activity in aqueous control) was in the order: Dichloromethane > Chloroform > Cyclohexane > Ethanol > 1,4-dioxane > DMSO > THF whereas that of HRP was Dichloromethane > Chloroform > Cyclohexane > Ethanol > DMSO > 1,4-dioxane > THF. The order of relative stability of the peroxidases also largely increased with the increasing polarity index of the organic solvents with cyclohexane as exception [22]. The activity of both RP and HRP was significantly lost in 20% THF, (Fig. 2(A)). The activity of RP remained always higher in 1,4-dioxane and ethanol at 0%–30% concentration, (Fig. 2(B) and (C)). Ethanol and 1,4-dioxane have almost similar polarity of 5.2 and 4.8, respectively [22]. In 10% 1, 4-dioxane and ethanol, RP exhibited 2 and 1.3 times higher activity respectively than HRP. In contrast HRP exhibited higher activity in DMSO, cyclohexane and chloroform (Fig. 2(D), (E), and (F)). The variation in activities of RP and HRP in hydrophobic organic solvents could possibly be due to multiple reasons including biphasic nature of the solvents or solvent-induced structural perturbations on the enzyme active site [23].

Unlike HRP, the RP showed a 1.3 times higher activity in 30% dichloromethane. This indicates that at a higher concentration of dichloromethane, RP behaves differently than HRP. Dichloromethane in aqueous medium can possibly result in a six-membered ring

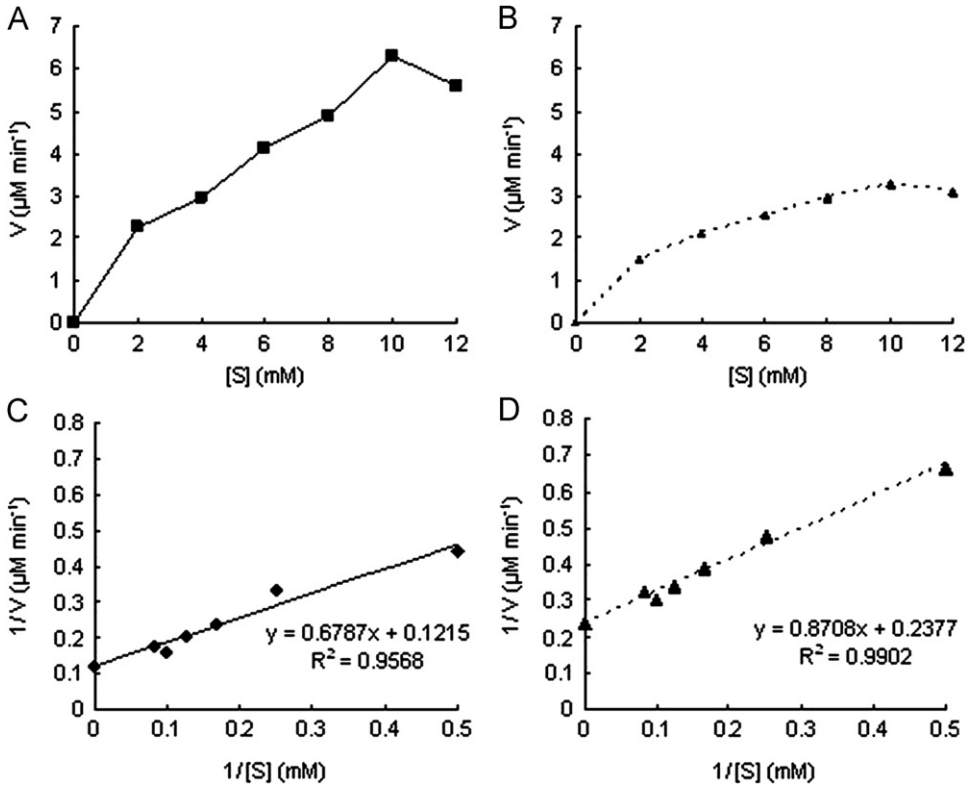


Fig. 1. Michaelis–Menten plot and Lineweaver-Burk double reciprocal plot for the effect of increasing concentrations of guaiacol on the initial velocity of RP (A), (C) and HRP (B), (D).

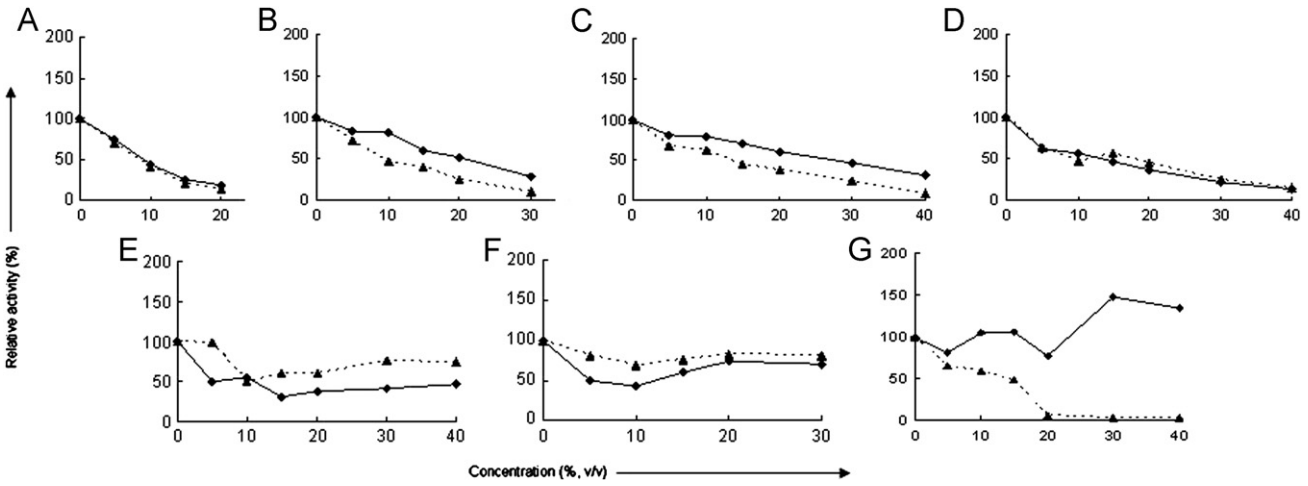


Fig. 2. Effect of varying concentrations of organic solvents. (A) THF, (B) 1,4-dioxane, (C) ethanol, (D) DMSO, (E) cyclohexane, (F) chloroform and (G) dichloromethane on % relative activity of RP (—) and HRP (– –) under standard assay conditions. The values are mean of three independent sets of experiments with SD of $< 5\%$.

structure, stabilizing the enzyme–solvent system. It is likely that in 30% dichloromethane, the structure or chemical nature of the hydrated RP enzyme and the enzyme–substrate complex is more ordered thereby providing better environment for activity of RP.

Exposure of peroxidase's active site to low or high volumes of water-miscible organic solvents alters the local polarity in the enzyme's active site and affects the transition state of the enzymic reaction and catalytic efficiency [23]. Crystal structure studies of peroxidase from higher plants including HRP and peanut peroxidase revealed that the unique aromatic region which is important for the ability of Class III peroxidases to bind to aromatic substrates is highly variable [24]. Therefore, the possibility of such a variation at the catalytic site of RP could also not be denied.

3.3. Shelf life of RP and HRP in varying concentrations of organic solvents

The activity of RP and HRP in varying concentration of 1, 4-dioxane, ethanol, THF and chloroform was studied for varied storage time at room temperature. A gradual decline with increasing storage time and concentration of solvents was noted with all the organic solvents used in the study. Fig. 3(A1)–(D1), and (A2)–(D2) shows the results of shelf life studies suggesting RP to be significantly stable till 60 h in 20% 1,4-dioxane and till 12 h in ethanol. The activity of RP and HRP increased gradually with 0%–40% ethanol as well as 0%–30% 1,4-dioxane till 20 h with a sharp decline thereafter (Fig. 3(A1), (A2) and (B1), (B2)). The trend

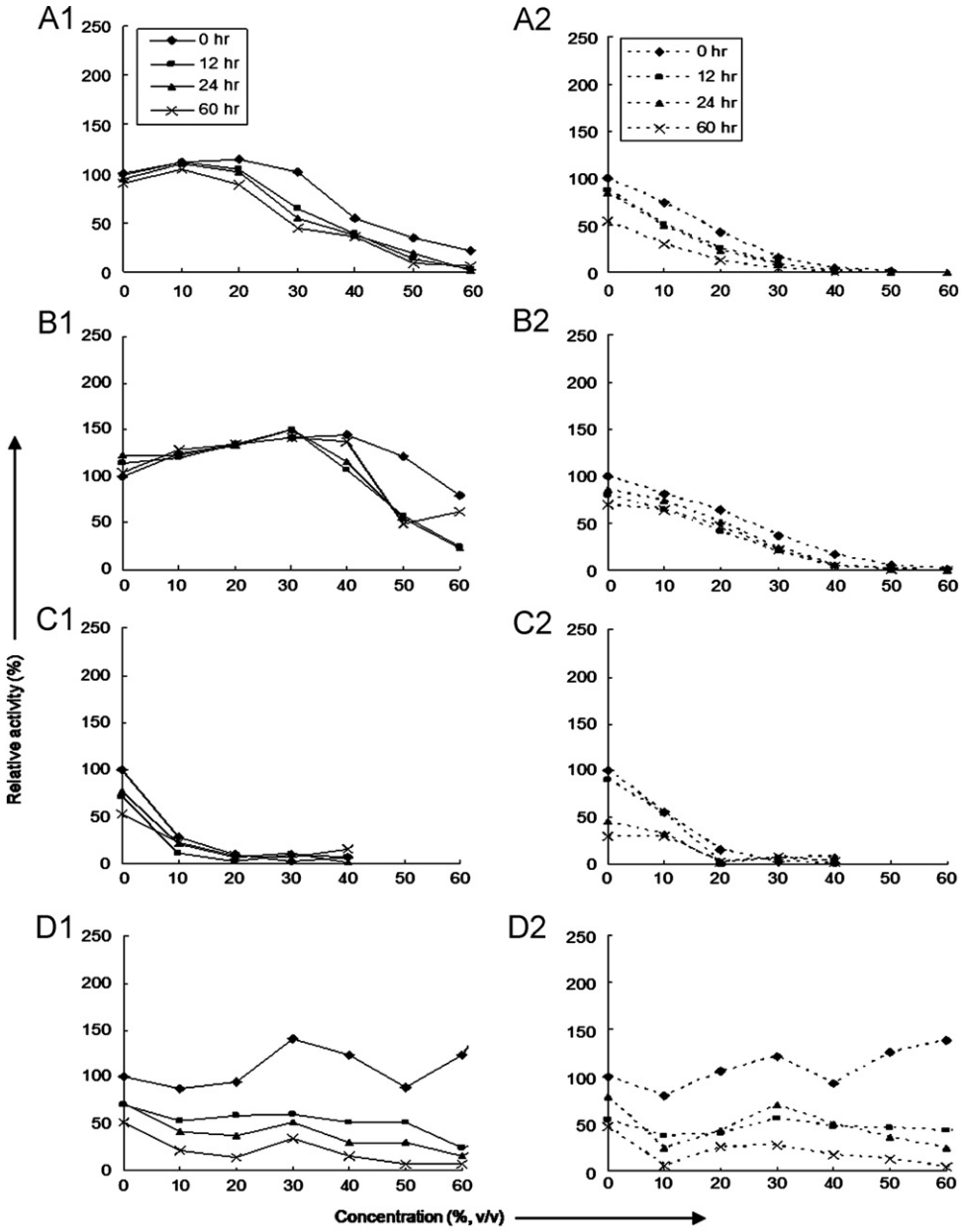


Fig. 3. Shelf life of RP (—) and HRP (– – –) in varying concentrations of organic solvents. (A1) and (A2)—1,4-dioxane, (B1) and (B2)—ethanol, (C1) and (C2)—THF and (D1) and (D2)—Chloroform. The enzyme activities were tested under standard assay conditions after storage of the enzyme in different solvent mixtures (% v/v) for the time indicated. The values are mean of three independent sets of experiment with SD of $<5\%$.

was in very good accordance with the findings of Khmelnitsky et al. [25] where numerous examples of enzyme (including oxidases and lipases) activation by moderate concentrations of 10%–30% organic solvents were drawn. RP had almost 40% loss in activity at 60 h whereas a complete loss in activity of HRP was noted under similar conditions (Figs. 3(A1), (A2) and (B1), (B2)). THF exerted most deleterious effect on enzyme activity wherein 20% THF led to almost 95% activity loss of both RP and HRP (Figs. 3(C1) and (C2)). In 30% concentration of chloroform ~98% activity of both RP and HRP was retained (Figs. 3(D1) and (D2)).

The enzyme stability reduced with increasing storage period nevertheless the stability of RP remained always higher than HRP with all concentration ranges of organic solvents. The results of the kinetic studies were tested using the intervals of confidence and agreed at 95% confidence level.

3.4. Kinetics of RP in organic solvents

Upon placing an enzyme in a non-aqueous medium, the biocatalyst is subjected to a number of factors that can alter its native, aqueous based, structure and function [23–28]. This may be the cause for appearance of larger K_m values in higher percent (v/v) concentrations of organic solvents.

Lee and Kim [29] proposed a theoretical kinetic model to describe enzyme reaction in organic solvents. Analysis showed that enzyme reaction rate in organic media depended largely upon the substrate solvation and enzyme hydration [29]. Any alteration in structure or chemical nature of the enzyme upon hydration leads to changes in Michaelis constant [26] whereas, the maximum reaction rate is independent of the medium composition [30,31]. This is because both, the activity coefficient

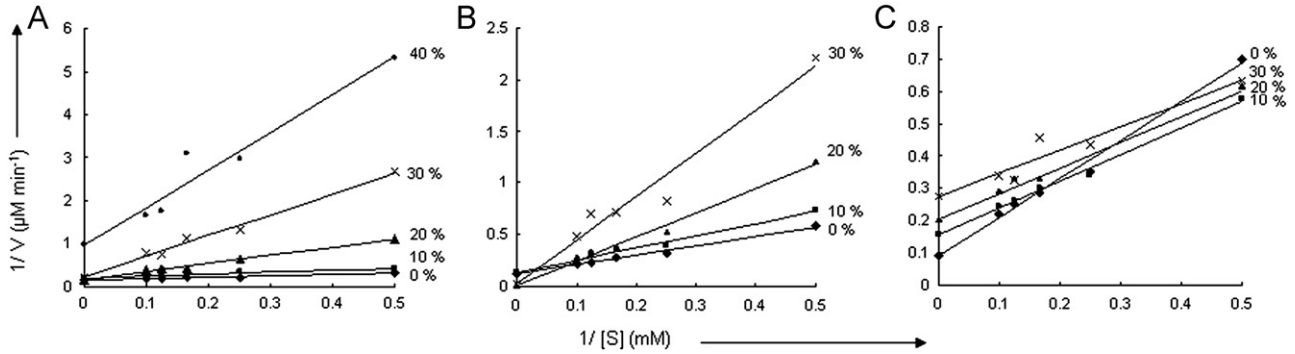


Fig. 4. Kinetic studies with rice peroxidase in different organic solvents. (A) 1,4-dioxane, (B) ethanol and (C) chloroform. The concentrations of the solvents (% v/v) in aqueous phosphate buffer is indicated in the figure.

Table 1
Kinetic parameters of RP as measured using 2, 4, 6, 8 and 10 mM substrate (guaiacol) concentrations in differing concentrations (% v/v) of organic solvents. The reaction media were set in phosphate buffer, pH 6.1. The enzyme activity was measured under standard assay conditions.

Organic solvent (% v/v)	Kinetic parameters								
	1,4-Dioxane			Ethanol			Chloroform		
	K_m	V_m	V_m/K_m	K_m	V_m	V_m/K_m	K_m	V_m	V_m/K_m
0	2.362	6.48	2.745	7.34	8.16	1.111	13.14	11.01	0.837
10	2.217	4.82	2.173	8.53	7.22	0.846	5.36	6.455	1.204
20	10.35	5.57	0.538	174	74.6	0.427	3.88	4.899	1.260
30	19.71	4.12	0.209	129	30.09	0.238	2.631	3.650	1.387
40	9.066	1.04	0.114	88.27	12.18	0.138	4.326	4.166	0.963

of the substrate and water together determine the rate of enzyme reaction in organic solvents. Fig. 4 shows the kinetics of RP in 1, 4-dioxane, ethanol and chloroform. Graphs clearly indicate that apparent Michaelis constant (K_m) values change largely as the reaction medium composition changes whereas the maximum reaction rate (V_{max}) is independent of the reaction medium in appropriate ranges of substrate concentrations. Therefore, instead of individual V_m and K_m, V_m/K_m is taken as the kinetic parameter to compare enzyme efficiencies in this study.

Table 1 summarizes the effect of varying substrate concentrations and % (v/v) concentration of organic solvents on K_m and V_m of RP. In water miscible organic solvents, *viz.*1,4-dioxane and ethanol, a regular decrease in V_m/K_m ratio can be seen with increase in organic solvent concentration whereas, a reverse trend was observed with water-immiscible solvent like chloroform. Enzymes that require less water molecules have a higher reaction rate in organic media [29]. This may be the case for RP where the reaction rate was in the order chloroform > ethanol > 1,4-dioxane (Fig. 4), which is in good accordance with the solvent polarity scale [22].

3.5. Immobilization studies with RP and HRP

The immobilization of both RP and HRP on poly-5-carboxy-indole was accomplished through ethyl-dimethylaminopropyl carbodiimide (EDC) and N-hydroxy-succinimide (NHS). The results of immobilization are shown in Fig. 5(A) and (B). The relative activity of RP and HRP enzymes upon immobilization shows an increasing enzyme activity with increase in time, with both guaiacol and dopamine hydrochloride as substrates, however the values were always higher for guaiacol than dopamine. This could be because of the amount of substrate used for enzyme assays. Experiment in our lab (data not included here) suggests

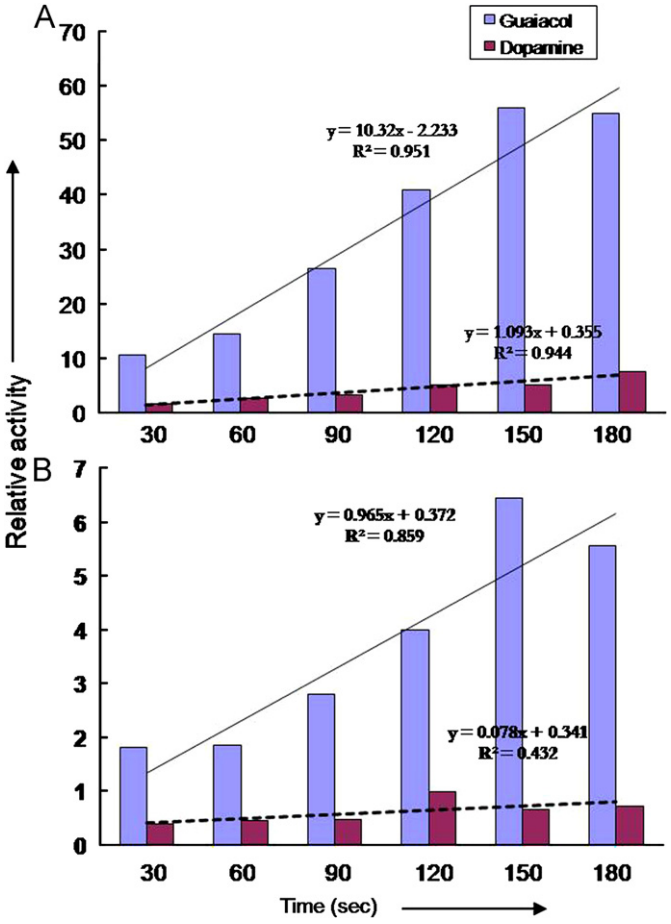


Fig. 5. Relative activity with increasing time for free (soluble) and immobilized RP (A) and HRP (B). Enzymes were immobilized on poly-5-carboxy-indole coated glass plates and assayed with 250 µL of 9 mM guaiacol or dopamine and 250 µL H_2O_2 (2 mM), under standard assay conditions.

optimum concentration for dopamine as substrate to be 50 mM, and that for guaiacol to be 9 mM. In order to equate the two substrates we had taken 9 mM concentration of both dopamine and guaiacol in the reaction mixture which is far below the experimental value obtained for dopamine. It is notable that immobilized RP had a better relative activity with dopamine as substrate than immobilized HRP, however for guaiacol both RP and HRP had a comparable activity upon immobilization.

To improve the stability of the desired biomolecules onto conducting polymers, several immobilization techniques including adsorption, entrapment, covalent attachment etc are used [32–37].

In this study adsorption technique was used to immobilize the biological component i.e. RP and HRP to the outer layer of the conducting polymer. This has been strengthened by free carboxyl groups present at the surface of the polymer that have been utilized for the covalent attachment of enzyme, peroxidase, through amide linkage with a carboxylic acid group, using the linkage reagents EDC and NHS. This step ensures the limitation of adsorption technique which suffers from the desorption of enzyme from the immobilizing material into the sample solution during measurement. As compared to the other immobilization methods, carbodiimide-coupling reaction has a feature of strong covalent bonding of enzyme with the matrix, which in turn is responsible for the high enzyme loading at the matrix surface [19]. It has been reported that functionalization of conducting polymer films provides suitable surface for covalent linkage of enzymes after carbodiimide activation [9].

4. Conclusion

This study has demonstrated the feasibility of rice peroxidase as a substitute for HRP in enzyme based applications including enzyme immobilization for developing a conducting polymer based biosensor. The applicability of rice peroxidase seems to be at par and even sometimes better than horseradish peroxidase making RP a cheaper and convenient enzyme system as HRP for immobilization using organic solvents. The high thermal stability and longer shelf life of RP over the immobilizing matrix and more stability in organic solvents show that the conducting polyindole having carboxyl functional groups can be utilized as a suitable matrix for the covalent entrapment of enzyme, rice peroxidase, through amide linkage. The low cost and simple method of immobilization is an additional advantage in fabrication of RP based electrode over conventional electrodes. A good sensitivity even at low substrate concentration and fast response to dopamine makes RP a suitable enzyme system for monitoring dopamine levels in aqueous medium. The experiments are presently in progress to fabricate and develop a conducting polyindole based covalently entrapped RP biosensor for measuring dopamine in biological fluids.

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References

[1] K. Yoshida, P. Kaothien, T. Matsui, A. Kawaoka, A. Shinmyo, Appl. Microbiol. Biotechnol. 60 (2003) 665–670.
[2] Y. Kulshrestha, Q. Husain, Enzyme Microb. Technol. 38 (2003) 470–477.
[3] A.M. Klibanov, Tu, T.M., K.P. Scott, Sci. 221 (1983) 259–261.
[4] J.A. Nicell, J. Chem. Technol. Biotechnol. 60 (1994) 203–215.
[5] J.S. Dordick, M.A. Marletta, A.M. Klibanov, Biotechnol. Bioeng. 30 (1987) 31–36.
[6] P. Dominguezsanchez, C.K. Osullivan, A.J. Mirandaordieres, P. Tunonblance, M.R. Smyth, Anal. Chim. Acta 291 (1994) 349–356.
[7] K.O. Lupetti, L.A. Ramos, I.C. Vieira, O. Fatibello-Filho, II Farmaco 60 (2005) 179–183.
[8] A.I. Gopalan, K.P. Lee, K.M. Manesha, P. Santosh, J.H. Kima, J.S. Kanga, Talanta 71 (2007) 1774–1781.
[9] B.D. Malhotra, A. Chaubey, S.P. Singh, Anal. Chim. Acta 578 (2006) 59–74.
[10] J.B. Spires, H. Peng, D.E. Williams, C. Soeller, J. Travas-Sejdic, Electrochim. Acta 55 (2010) 3061–3067.
[11] D.R. Hoagland, D.I. Arnon, Calif. Agric. Exp. Stn. Circ. 3 (1938) 346–347.
[12] K. Shah, R.G. Kumar, S. Verma, R.S. Dubey, Plant Sci. 161 (2001) 1135–1144.
[13] C. Penel, H. Greppin, Plant Physiol. Biochem. 34 (1996) 479–488.
[14] S. Nahakpam, P. Singh, K. Shah, Biotechnol. Bioprocess Eng. 13 (2008) 632–638.
[15] G.H. Egley, R.N. Paul Jr., K.C. Vaughn, S.O. Duke, Planta 157 (1983) 224–232.
[16] P. Nagaraja, K.C. Srinivasa Murthy, K.S. Rangappa, N.M.M. Gowda, Talanta 46 (1998) 39–44.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[18] Jian-Zhong Liu, Teng-Li Wang, Ming-Tao Huang, Hai-Yan Song, Li-Ping Weng, Liang-Nian Ji, Protein Eng. Des. Sel. 19 (2006) 169–173.
[19] V. Rajesh, W. Bisht, K. Takashima, Kaneto, React. Funct. Polym. 62 (2005) 51–59.
[20] K. Ryu, Y. Kim, Biotechnol. Lett. 19 (1997) 1019–1022.
[21] K.G. Welinder, Curr. Opin. Struct. Biol. 2 (1992) 388–393.
[22] C. Reichardt, Solvents and Solvent Effects In Organic Chemistry, 3rd ed., Wiley -VCH Publishers, 2003, pp. 57–91.
[23] K. Ryu, J.S. Dordick, Biochem. 31 (1992) 2588–2598.
[24] M. Gajhede, David J. Schuller, A. Henriksen, Andrew T. Smith, Thomas L. Poulos, Nat. Struct. Mol. Biol. 4 (1997) 1032–1038.
[25] Y.L. Khmelnitsky, L.V. Levashov, N.L. Klyachko, K. Martinek, Enzyme Microb. Technol. 10 (1988) 710–724.
[26] S.J. Singer, Adv. Protein Chem. 17 (1962) 1–68.
[27] C. Tanford, Physical Chemistry of Macromolecules, Wiley, New York, 1961, pp. 16–179 (Chapter 6).
[28] G.E. Schultz, R.H. Schirmer, Principles of Protein Structure, Springer-Verlag, New York, 1979, p. 314.
[29] S.B. Lee, K.-J. Kim, J. Ferment. Bioeng. 79 (1995) 473–478.
[30] K. Martinek, A.V. Levashov, N.L. Klyachko, V.I. Pantin, I.V. Berezin, Biochim. Biophys. Acta 657 (1981) 277–294.
[31] H. Nakajima, K. Suzuki, K. Imahori, Agric. Biol. Chem. 49 (1975) 317–323.
[32] M. Gerard, A. Chaubey, B.D. Malhotra, Biosens. Bioelectron. 17 (2002) 345–359.
[33] S. Cosnier, Anal. Bioanal. Chem. 377 (2003) 507–520.
[34] S. Cosnier, D. Fologea, S. Szunerits, R.S. Marks, Electrochem. Commun. 2 (2000) 827–831.
[35] S. Cosnier, A.L. Pellec, R.S. Marks, K. Perie, J.-P. Lellouche, Electrochem. Commun. 5 (2003) 973–977.
[36] V. Rajesh, W. Bisht, K. Takashima, Kaneto, Surf. Coat. Technol. 198 (2005) 231–236.
[37] P. Tengvall, E. Jansson, A. Askendal, P. Thomsen, C. Gretzer, Colloids Surf. B: Bioinf. 28 (2003) 261–272.