Immobilization of Invertase in Conducting Polypyrrole/ PMMA-*co*-PMTM Graft Copolymers

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ABSTRACT: In this study, invertase was immobilized in copolymer electrodes constructed. Three different types of polymethyl methacrylate-*co*-polymethyl thienyl methacrylate matrices were used to obtain copolymers that were characterized by FT-IR spectroscopy. Immobilization of enzymes was carried out by the entrapment of the enzyme in conducting polymer matrices during electrochemical polymerization of pyrrole through thiophene moieties of polymers. Immobilization of the enzyme was achieved by application of 1.0 V constant potential on a platinum electrode for 30 min in solution. The effects of temperature and pH on the activity of the enzyme electrodes were examined and operational stability studies were done. The changes in the maximum reaction rate and the variations in the Michaelis–Menten constant were studied. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 502–507, 2005

Key words: polypyrroles; conducting polymers; enzymes; macromonomers

INTRODUCTION

Enzymes are biological catalysts that increase the rate of chemical reactions taking place within living cells by lowering the energy of activation, without themselves appearing in the reaction products. Unlike most inorganic catalysts, enzymes are generally soluble and unstable; thus, these organics can be used only once in free solutions. Enzymes may be used in industry in free or immobilized forms.[1]

Fixation of an enzyme to the surface of the carrier by adsorption or by covalent attachment or by entrapment of enzymes within a polymeric matrix by membrane confinement or liposome techniques are the most widely used methods for immobilization of enzymes. Immobilization can affect the stability, pH and the temperature optima, the Michaelis–Menten constant (Km), and the maximum reaction rate (Vmax) of an enzyme. These depend on the methods of immobilization and the nature of the carrier.[2]

Polymerization based on electrochemical oxidation of a given monomer, from a solution containing enzyme, is one of the alternative methods of enzyme immobilization in polymer at the working electrode surface, and results in formation of a conducting polymer layer containing entrapped enzyme molecules. The electropolymerization is often done in aqueous solutions of pH close to neutral values to immobilize the enzyme without loss of activity.[3]

Immobilization of enzymes makes heterogeneous catalysis possible, which has great advantages: it is possible to use a single batch of enzymes repetitively and to stop the reaction by physical removal of the immobilized enzyme from the solution. Also, in many cases, the enzyme is stabilized by bonding. Additional advantages include easy analyte determination in complex mixtures and use of small sample volumes. The enzyme will still be active and largely uncontaminated, so it can be used again. Also, due to the longer life, predictable decay rate and elimination of reagent preparation are further advantages of immobilization.[4,5]

Invertase, known as β-froctofuranosidase (E.C. 3.2.1.26), catalyzes the hydrolytic breakdown of sucrose to glucose and fructose. The mixture of these products has a lower crystallinity than sucrose at high concentrations and does not crystallize out like sucrose. The usage of invertase confectionary thus ensures that the products remain fresh and soft even when kept for a long time. Therefore, it is widely used in the production of artificial honey and to a small extent in the industrial production of liquid sugar.[6] The immobilized form of invertase has so far only been employed experimentally, since the soluble enzyme is available at little cost; however, the use of invertase in the entrapment process sheds light on the immobilization of expensive enzymes in conducting polymer matrices.

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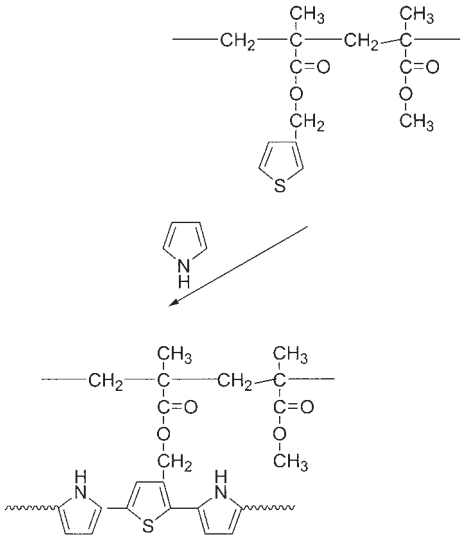

Scheme 1 Synthesis of conducting copolymer of PMMA-*co*-PMTM/PPy.

In this study, the immobilization of invertase was done via entrapment within three types of polypyrrole/PMMA-*co*-PMTM matrices (Scheme 1). The types of PMMA-*co*-PMTM random copolymer coded as MT1, MT2, and MT3 have the same segments but they have different copolymer compositions with respect to mole percents (Table I). These random copolymers were synthesized and characterized previously.[7] The conducting copolymers with pyrrole were synthesized using sodium dodecyl sulfate (SDS) as the supporting electrolyte and characterized via IR spectroscopy. Then, optimum conditions for these electrodes, such as pH, temperature, and kinetic parameters (Km and Vmax), were investigated. The operational stability was also studied.

EXPERIMENTAL

Apparatus

For the electrochemical synthesis, a potentioscan Wenking POS-73 model potentiostat, a Shimadzu model FT-IR spectrophotometer for the characterization of conducting copolymers, and a Shimadzu UV-1601 model spectrophometer for enzyme activity measurements were used.

Materials

Invertase, β-froctofuranosidase (E.C. 3.2.1.26), and sodium dodecyl sulfate (SDS) were purchased from Sigma. Pyrrole (Merck) was distilled and stored at 4°C. For the preparation of the Nelson reagent, sodium carbonate (Riedel de Haen), sodium potassium tartarate (Riedel de Haen), sodium bicarbonate (Merck), and sodium sulfate (Merck), and for the preparation of the arsenomolybdate reagent; ammonium heptamolybdate (Merck) and sodium arsenate (Merck) were used as received.

Synthesis of MT1/PPy, MT2/PPy, and MT3/PPy conducting copolymers

1% solutions of (w/v) of MT1, MT2, and MT3 were prepared in dichloromethane. The thickness of these electrodes was ca 8–10 μm in terms of polymers. Pyrrole was polymerized electrochemically on platinum (Pt) electrodes that were previously coated with MT1, MT2, and MT3. SDS was used as the supporting electrolyte in water, and electropolymerization yielded a black film on the electrode after 30 min of reaction by applying 1.0 V against the Ag/Ag^+ reference electrode.[7,8] Blank experiments with no pyrrole in the electrolysis medium were performed to check whether MT polymers can degrade or not under the applied potential.

Immobilization of invertase in MT1/PPy, MT2/PPy, and MT3/PPy conducting copolymers

Electropolymerization was performed in a cell, consisting of the Pt as the working electrode, 10 mL acetate buffer (50 mM pH = 5.0) solution containing 0.6 mg/mL invertase, 0.6mg/mL SDS, and 40 μL pyrrole. The working electrode was coated with MT1/MT2/MT3 from its dichloromethane solution. Electrolyses were done at 1.0V constant potential on the potentiostat for 30 min.

Determination of enzyme activity

For immobilized invertase, different concentrations of sucrose in buffer solution (acetate buffer pH = 5.0) were placed in test tubes and put in a water bath at 25°C for 10 min. After preincubation, enzyme electrodes were immersed in test tubes and shaken in the water bath for 2–6 min.

Aliquots (1.0 mL) of these solutions were withdrawn, and 1.0 mL Nelson's reagent was added.[9] The tubes were then placed in boiling water for 20 min. Samples were cooled in an ice-bath, and 1.0 mL of arsenomolybdate solution was added to the tubes and mixed by vortexing. Finally, 7.0 mL of distilled water

TABLE I
Random Copolymers

Code	Mn	Copolymer composition (mol %)	
		PMTM	PMMA
MT1	1.1×10^5	17	83
MT2	1.1×10^5	25	75
MT3	1.2×10^5	45	55

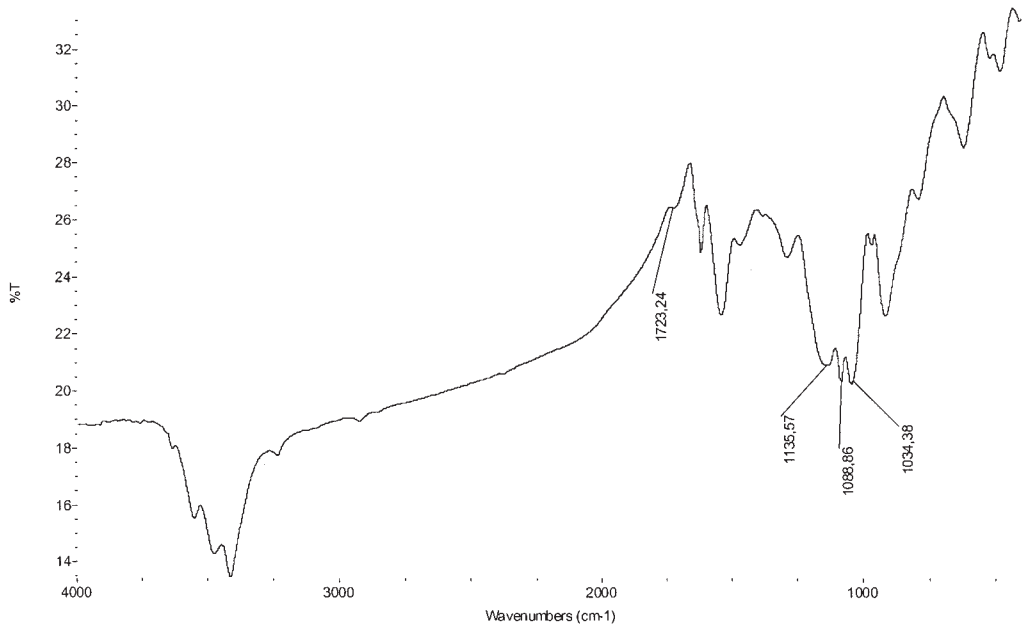

Figure 1 IR spectrum of conducting copolymer of PMMA-*co*-PMTM/PPy.

was added to each of the test tubes. After mixing, absorbance was measured at 540 nm using a Shimadzu UV-1601 model spectrophotometer.

Determination of optimum pH and optimum temperature

The reaction temperature was changed between 10°C and 80°C while sucrose concentration was kept constant at about 10 Km for each system. For pH optimization at 25°C, the pH of the reaction was changed between pH 2 and pH 9 while sucrose concentration was kept constant at about 10 Km for each system. The activities were determined as previously described.

RESULTS AND DISCUSSION

Characterization of MT1/PPy, MT2/PPy, and MT3/PPy conducting copolymers

The spectra of the pristine random copolymers reveal thienylene C-Hα stretching at 3111 cm^{-1}, aliphatic C-H stretching at 2995–2950 cm^{-1}, carbonyl group at 1729 cm^{-1}, C═C stretching at 1447 cm^{-1}, C-O-C stretching at 1148–1241 cm^{-1}, and thienylene stretching at 786 cm^{-1}.

FTIR spectra of SDS doped MT1/PPy, MT2/PPy, and MT3/PPy showed peaks at 3400, 1440, and 1124 cm^{-1} due to C-N and C-C stretching, which are characteristic peaks for PPy. A characteristic peak belonging to the carbonyl group of a pristine random copolymer was also observed at 1723 cm^{-1}. The peaks between 1200 and 1034 cm^{-1} represent the presence of SDS anion. These results indicate that the copolymers of MT1/PPy, MT2/PPy, and MT3/PPy occurred (Fig. 1).

Kinetic parameters of immobilized invertase

Vmax and Michaelis–Menten constants (Km) for enzyme electrodes were found from a Lineweaver–Burk plot.[10] Kinetic constants for immobilized invertase in three matrices are given in Table II. Although Km and Vmax values of MT3/PPy and MT2/PPy are close to each other, Km and Vmax values of MT1/PPy were greater than those values of MT3/PPy and MT2/PPy and they were close to Km and Vmax values of the PPy matrix. This event can be explained as the thienyl group amount in the MT1 is the lowest among three of the random copolymers and more polypyrrole chains are formed in the MT1/PPy copolymer matrix compared to the MT3/PPy and MT2/PPy matrices. Due to this, the MT1/PPy matrix may show properties very similar to those of PPy. In addition, Km is a parameter that is inversely proportional to the affinity of enzyme to a substrate. Large Km indicates that substrate and

TABLE II
Kinetic Parameters for Free and Immobilized Invertase

	Km (mM)	Vmax (μmol/min)
Free Invertase	24.3	82.3
PPy/Invertase	58.0	3.0
MT1/PPy/Invertase	54.0	4.0
MT2/PPy/Invertase	8.4	1.2
MT3/PPy/Invertase	8.3	1.0

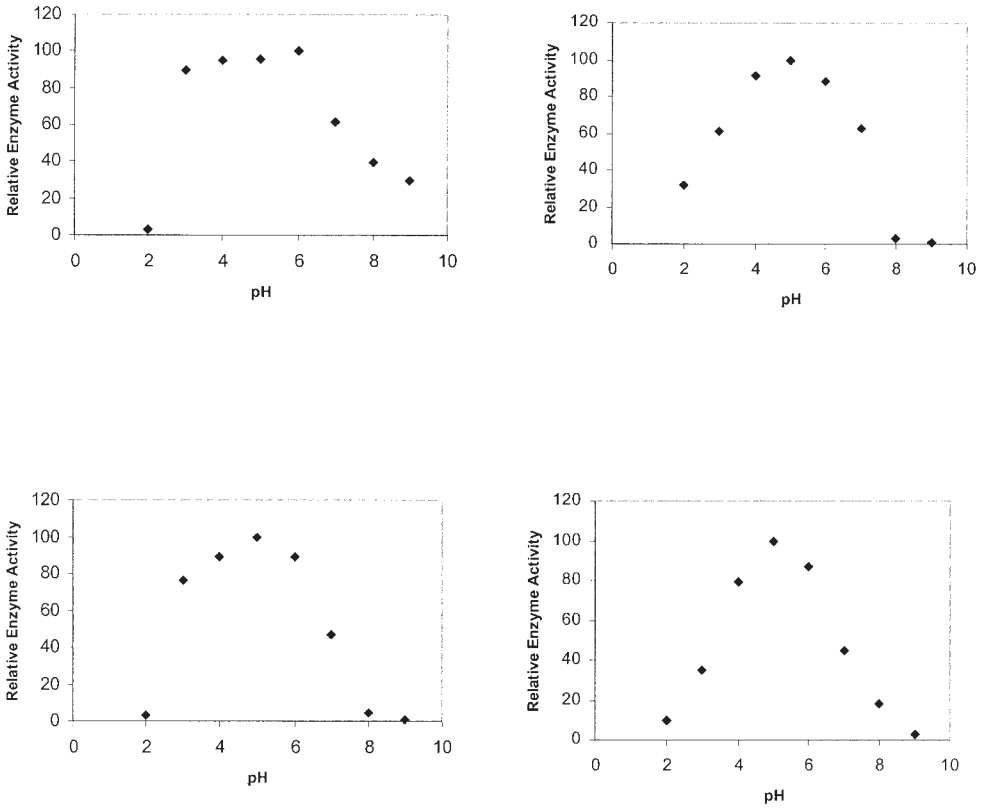

Figure 2 Effect of pH on invertase activity immobilized in (a) MT1/PPy; (b) MT2/PPy; (c) MT3/PPy; (d) PPy.

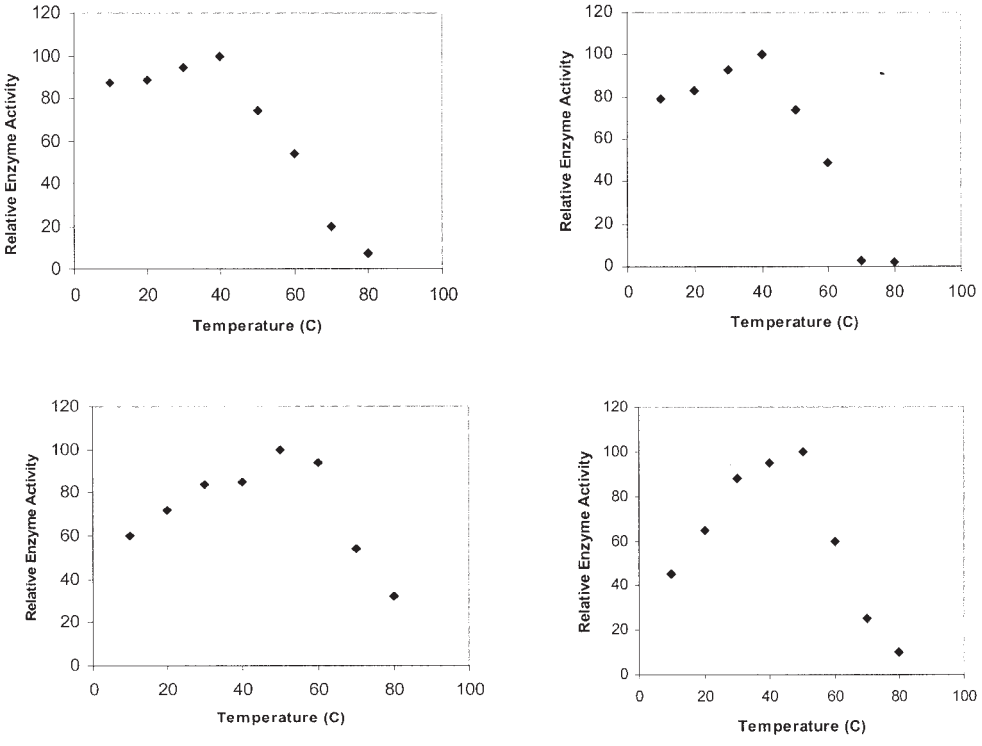

Figure 3 Effect of incubation temperature on invertase activity in (a) MT1/PPy; (b) MT2/PPy; (c) MT3/PPy; (d) PPy.

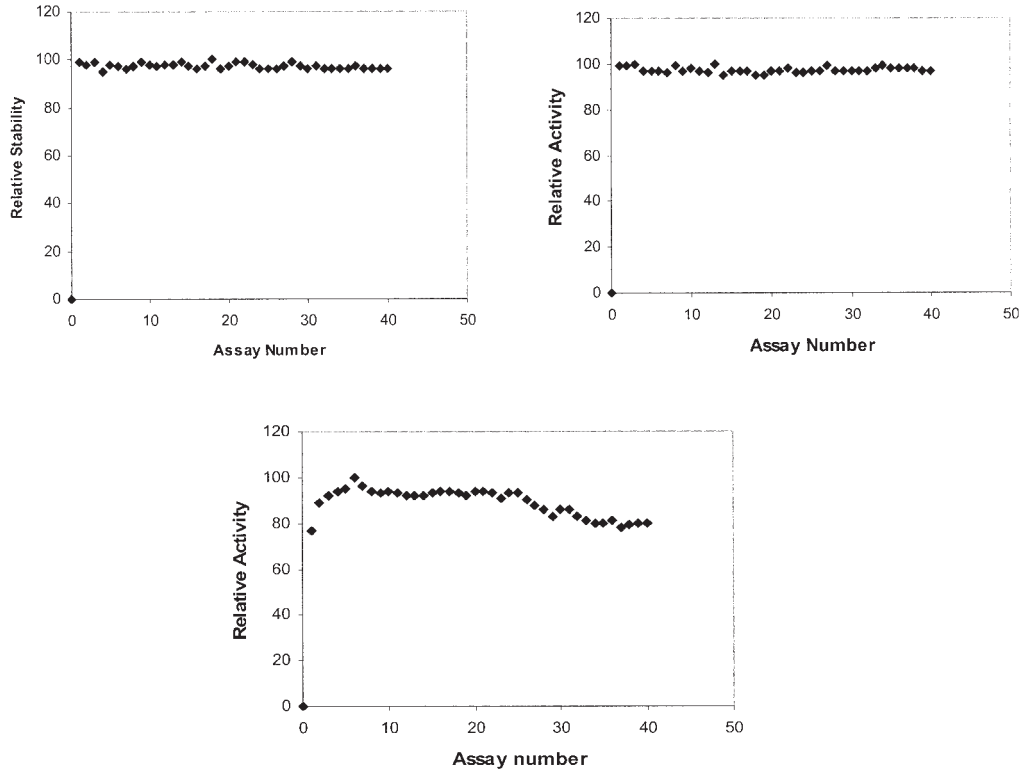

Figure 4 Operational stability of (a) MT1/PPy; (b) MT2/PPy; (c) MT3/PPy.

enzyme do not prefer to be close for a long time. The MT1/PPy matrix exhibits higher Km values than those of the MT3/PPy and MT2/PPy matrices. This may be also another explanation of the higher Vmax value of invertase entrapped in MT1/PPy than those of the MT3/PPy and MT2/PPy matrices. The substrate produces the product and immediately leaves each other to give way for the next substrate.[11] Another important point is that MT2/PPy and MT3/PPy exhibit a smaller Km value than free invertase. A smaller Km value than free enzyme[12] indicates that this matrix provides a microenvironment that is more suitable than the one in the solution.

Influence of pH on the enzyme activity

Enzymes are often assayed at their optimal pH. An enzyme's apparent response to pH may change dramatically when it is in a heterogeneous environment associated with polymer matrices. The study of such changes and the factors influencing them are of great interest not only to those people wishing to learn more of the way in which enzymes work *in vivo*, but also the biochemical engineer who also needs to be aware of all of the factors that may influence the activity of an immobilized enzyme preparation. During the immobilization of invertase in conducting graft copolymer matrices, protons are released into the electrolysis media during the electrochemical polymerization of pyrrole. This causes the pH of the medium to decrease. Changes in pH can affect the enzyme structure and also cause denaturation. To prevent this, a buffer solution must be used. In these experiments, the pH of the buffer was 5.0. The maximum activity was obtained at pH 4.6 for the free enzyme.[13] The maximum pHs were found to be 5.0 for the MT3/PPy and MT2/PPy matrices and 6.0 for the MT1/PPy matrix; they are illustrated in Figures 2a–d. The optimum pHs were shifted towards the alkaline side when compared with the free enzyme. This might be explained by partitioning of protons. Negatively charged groups of the matrix will tend to concentrate protons, and this causes lowering the pH around the enzyme. Therefore, the pH around the enzyme will be lower than that of the bulk phase from which the measurement of pH is carried out. In addition to these, although the pH responses of the MT3/PPy and MT2/PPy matrices were alike, the response of MT1/PPy against pH variation was different. The MT1/PPy matrix gave the highest stability to lower pHs, such as pH 3–5. Because of this, the MT1/PPy matrix can be used reliably at low pH values for enzyme reactions.

Effect of temperature on enzyme activity

The effect of temperature between 10 and 80°C on the relative enzyme activity was investigated, and it is illustrated in Figures 3a–d. The maximum tempera-

ture for the free enzyme was found to be 50°C.[14] Maximum enzyme activity for the MT2/PPy and MT1/PPY matrices was at 40°C, and for MT3/PPy it was at 50°C. Although the MT2/PPy and MT1/PPY matrices at low temperatures showed higher stability, at high temperatures the enzyme activity for both matrices reduces rapidly. The opposite behavior was recorded for the MT3/PPy matrix.

Operational stability

We tried to estimate the stability of the electrodes in terms of repetitive uses. In 40 successive measurements, we observed very small fluctuations for all matrices. The results are shown in Figures 4a–c. The enzyme activities were almost stable during 40 experiments performed at 25°C in 1 day.

CONCLUSIONS

In this study, invertase was successfully immobilized in conducting PPy/PMMA-*co*-PMTM matrices. The changes in pH, temperature, and kinetic constant upon immobilization and upon incorporation of PMMA-*co*-PMTM into the PPy matrix were investigated. The changes among them and as compared to free invertase and PPy were observed. These data show that conducting copolymers can be used for the immobilization of invertase.

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