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Immobilization of Invertase in a Novel Proton Conducting Poly(vinylphosphonic acid) – poly(1-vinylimidazole) Network

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A novel proton conducting polymer blend was prepared by mixing poly(vinylphosphonic acid) (PVPA) with poly(1-vinylimidazole) (PVI) at various stoichiometric ratios via changing molar ratio of monomer repeating unit to achieve the highest protonation. The polymer network having the most suitable stoichiometric ratio for substantial proton conductivity was prepared and characterized by FT-IR spectroscopy and proton conductivity measurements. The network was used for immobilization of invertase and some important kinetic parameters such as the maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) were investigated for the immobilized invertase. Additionally, optimum temperature and pH were determined to acquire the best conditions for the highest enzyme activity. Operational stability of the entrapped enzyme was also examined. The results reveal that the most stable and highly proton conducting polymer network may play a pioneer role in the biosensors applications as given by FT-IR, elemental analysis, impedance spectroscopy and storage stability experiments.

Keywords: Poly(vinylphosphonic acid), poly(1-vinylimidazole), invertase, enzyme immobilization, proton conductivity

1 Introduction

Poly(vinylphosphonic acid) (PVPA) is a polymer with a proton conduction capacity. Although PVPA has two acidic protons, it behaves like a monoprotic acid since it is almost impossible to use the second acidic proton due to electrostatic interaction. PVPA includes a high concentration of phosphonic acid groups, and offers a good model system to study the correlation between the structure and proton conduction mechanism. Besides, PVPA alone is a very rigid material due to the existence of a high concentration of phosphonic acid groups, which are bonded by hydrogen bridges to each other (1). The choice of the proper protogenic group plays an important role in the proton conductivity of polymer electrolyte materials. The ideal protogenic group should demonstrate proton donor and acceptor properties. It should tend to form intermolecular hydrogen bonds like N-heterocycles (2). Heterocycles such as imidazole behaves as proton acceptors through the nitrogen side which forms protonic charge carriers (3). Poly(1-vinylimidazole) (PVI) is a common polymer to work with since (i) it can be synthesized in a simple way; (ii) it is a polybase and its positive charge density is pH dependent; (iii) its imidazole group have complexation properties; (iv) it is a weak basic polyelectrolyte to study adsorption properties on various minerals (4).

A polymer electrolyte membrane fuel cell (PEMFC) is an electrochemical cell which converts the chemical energy of a fuel and the oxidant to electrical energy continuously (5). The proton exchange membrane provides ionic transport between anode and cathode compartments of the fuel cell. Nafion® as a membrane material for low-temperature fuel cell applications show high proton conductivity depending on the presence of water. Also, its appreciable chemical and thermal stability makes it a standard material for fuel cell applications. On the other hand, there are several drawbacks for practical use of Nafion; high cost, maximum operation temperature, problems associated with the transport of water and recycling of the perfluorinated material (6). However, phosphonic acid based systems offer the possibility to obtain high proton conductivities over a wide temperature range between room temperature up to 200°C (7). In this respect poly(1-vinylimidazole) was used as the proton acceptor species and doped with poly(vinylphosphonic acid) for the formation of proton conducting polymer network.

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Biological response of the biosensor is determined by the biocatalytic membrane which accomplishes the conversion of reactant to product. Enzymes are frequently used as biocatalysts in biosensors. An enzyme is a substance that catalyzes the chemical reactions in living organisms without exposing any change while regulating rate of the proceeding reactions (8). Immobilized enzymes show a number of advantageous features which makes them particularly applicable for use in biosensor technology. Several enzyme immobilization techniques have been developed throughout the immobilization studies which are carrier binding (ionic binding, covalent bonding physical adsorption), crosslinking, adsorption or entrapment methods (9).

Invertase α-Fructofuranosidase (E.C. 3.2.1.26) is one of the most widely used industrial enzymes. It is also known as invert sugar and catalyzes the hydrolysis of sucrose to glucose and fructose. In sweet industry invertase is widely used in the production of artificial honey and also production of liquid sugar. In the literature it has been reported that immobilization of invertase has been achieved on conducting polymers (10), carbohydrate moieties (11) and polyelectrolytes (12).

In the present study, a novel polymer electrolyte network with a very high stability was achieved. PVPA and PVI mixtures with different mixing ratios were characterized to get the most suitable network for the entrapment of invertase. Furthermore, crucial enhancement in invertase stability and high electrolyte resistance to various conditions were confirmed with the enzyme kinetic studies.

2 Experimental

2.1 Reagents

Vinylphosphonic acid, and $\alpha\alpha'$azodiisobutyramidin dihydrochloride (ABADH) were purchased from Fluka. Invertase α-fructofuranoxidase) (E.C 3.2.1.26) and sucrose were purchased from Sigma and used as received without further purification. For the preparation of Nelson Reagent, sodium carbonate, sodium potassium tartarate, sodium bicarbonate, sodium sulfate, copper sulfate pentahydrate were purchased from Aldrich. Arsenomolibdate reagent was prepared by mixing ammonium heptamolibdate tetrahydrate (Aldrich) with sodium hydrogen arsenate dibasic 7-hydrate (Aldrich). For the preparation of phosphate, acetate and tris buffers, sodium phosphate monobasic, sodium phosphate dibasic, sodium acetate trihydrate, acetic acid and tris base were purchased from Sigma.

2.2 Instrumentation

For the determination of enzyme activity a Shimadzu UV–1601 model spectrophotometer was used. The FTIR spectra of proton conducting polymer blends with different mixing ratios were measured using Varian 1000 FTIR Spectrometer as KBr pellets. Elemental analysis measurements were performed by LECO, CHNS-932 device. AC conductivities of the proton conducting blends were measured by Novocontrol Alpha Dielectric Analyzer. Impedance measurements were carried out to determine frequency dependent proton conductivity of the samples as a function of temperature. Prior to measurements, samples were dried under vacuum at 60°C for 24 h. Thermal gravimetry analysis of the polymer network was investigated by Perkin Elmer STA 6000 instrument with a heating rate 10°C/min. For differential scanning calorimetry (DSC) measurements, a Perkin-Elmer JADE DSC instrument was used. Shimadzu UV-160-A model spectrophotometer was used for spectrophotometric analysis.

2.3 Preparation of Enzyme Immobilized PVPA/PVI Polymer Network

PVPA synthesis was achieved via free radical polymerization of vinylphosphonic acid using ABADH as the initiator. Polymerization occurs via cyclopolymerization of the vinylphosphonic acid anhydride as an intermediate (1). On the other hand, synthesis of PVI was achieved by solution polymerization (13). To achieve PVPA/PVI matrix, 0.0115gr of PVPA were mixed with different amounts of PVI. Three different complexes with distinct ratios were prepared as 1:1; 1:2; 1:3 by varying the amount of PVI as 0.01; 0.02; 0.03 g, respectively. PVPA behaves as a monoprotic acid hence; the maximum protonation was expected at 1:1 ratio (Fig. 1). This refers to the molar ratio of proton donor and acceptor groups. (1-Vinylimidazole)acts as the proton acceptor group in PVI whereas POOH acts as the proton donor group in PVPA. The best mixing ratio was characterized in terms of maximum degree of complexation, physical stability and proton conductivity with the aid of FTIR, TGA, DSC, elemental analysis and impedance measurements. The most suitable polymer blend (1:1 ratio) has been formed by complexation of 0.046 gr/mL PVPA with 0.04 gr/mL PVI. Invertase (4 mg/ mL, 107 U) was immobilized in the network and this ratio was used throughout the experiments.

2.4 Determination of Invertase Activity

The activity of invertase was determined with Nelson's method (14). Different concentrations of sucrose solution in acetate buffer (pH 5.5) were prepared. Different incubation times (2, 4, and 6 min) were applied to allow enzymatic reaction in a total volume of 4.0 ml of substrate solution. Solutions were kept in a water bath for 5 min to achieve temperature equilibrium, and then the enzyme immobilized network was placed in the test tubes and shaken for incubation times of 2, 4, 6 min. After incubation time, 1 ml of this solution was taken and added into 1 ml Nelson reagent and put into boiling water for 20 min to terminate the enzymatic activity completely, and finally solutions were

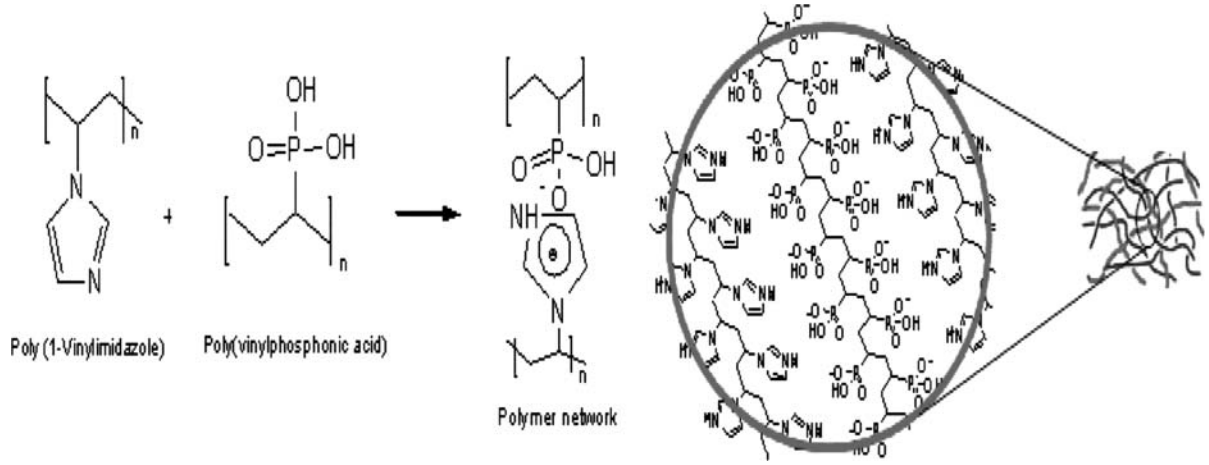

Fig. 1. Protonation of PVI with PVPA.

cooled to room temperature. After cooling, 1.0 ml of arsenomolybdate solution and 7.0 ml of distilled water were added for spectrophotometric measurements. Absorbance measurements were done at 540 nm. The immobilized enzymes were kept at 4°C in acetate buffer when not in use.

2.5 Determination of Optimum Temperature and pH

To determine the optimum temperature, medium temperature was changed between 10°C and 80°C, while keeping the substrate concentration constant. The effect of pH was determined by changing pH between 5.0 and 9.0 at constant temperature and substrate concentration. Acetate buffer was used for pH 5, 5.5; phosphate buffer was used for pH 6, 6.5, 7, 7.5 and tris buffer was used for pH values 8, 8.5 and 9.

2.6 Operational, Storage and Thermal Stability Experiments

The operational stability experiments were performed by 25 subsequent activity measurements in the same day at pH 5.5 and 25°C. The storage stability of the immobilized invertase was investigated by performing activity measurements within 75 days. Thermal stability experiments were carried out at 30, 40, 50, 60°C and pH 5.5 using different immobilized enzymes in 25 activity assays for each temperature. Moreover, for the further enhancement of operational stability, stability experiments were performed at the optimum temperature and pH where enzyme shows maximum activity.

3 Results and Discussion

3.1 FT-IR Results

FT-IR spectra of PVPA-PVI polymer complexes with different mixing ratios were analyzed and compared. A strong absorption peak at 1150 cm^{-1} belongs to P=O stretching. Relatively broad peak appeared between 1040–910 cm^{-1} represents the (P-O)-H stretchings of phosphonic acid groups of the PVPA. The phosphonic acid group gives a band in the region 1700–1630 cm^{-1}. Additionally, the broad band at 3300–2850 cm^{-1} belongs to OH stretchings. After blending PVPA with PVI in different ratios (1:1, 1:2, 1:3), the intensity of the P=O stretching at 1150 cm^{-1} decreased and the one for (P-O)-H became stronger due to the protonation of PVI. These occur by the transfer of the acidic proton of phosphonic acid to the 'free' nitrogen side of PVI to form imidazolium ion (15). The absorption band near 3150 cm^{-1} corresponds to the N-H group and appears relatively stronger in 1:1 and 1:2 mixing ratios. Additionally, the weak absorption band at 1590 cm^{-1} was attributed to the protonated heteroaromatic ring. Moreover, hydrogen bond formation between aryl-N and H of phosphonic acid group of PVPA results in the broadening of the 2800–2600 peak and a new N-H stretching appears at 3400–3200 cm^{-1} (16) PVPA-PVI (1:1) and (1:2) polymer blends have been determined as successful complex polymers in terms of proton transfer (Fig. 2).

3.2 Elemental Analysis

Three different (ratios; 1:1, 1:2, 1:3) polymer blends were prepared and dried samples were subjected for C, H and N analyses for all polymers. Prior to elemental analyses, complexes with different mixing ratios were dried under vacuum for 3 days. Table 1 show that percent ratios of PVI vary between 50 and 75 in the feed, whereas percent PVI content of the complexes changes between 58 and 63. This study also reveals that the polymer complex with 1:1 mixing ratio has higher PVI content (58.06%) than the expected value (50%). In this sense, PVPA-PVI (1:1) blend turned out to be the one with the highest physical stability. Hence, other characterization experiments were performed on PVPA-PVI (1:1). In recent work, PVPA was reported

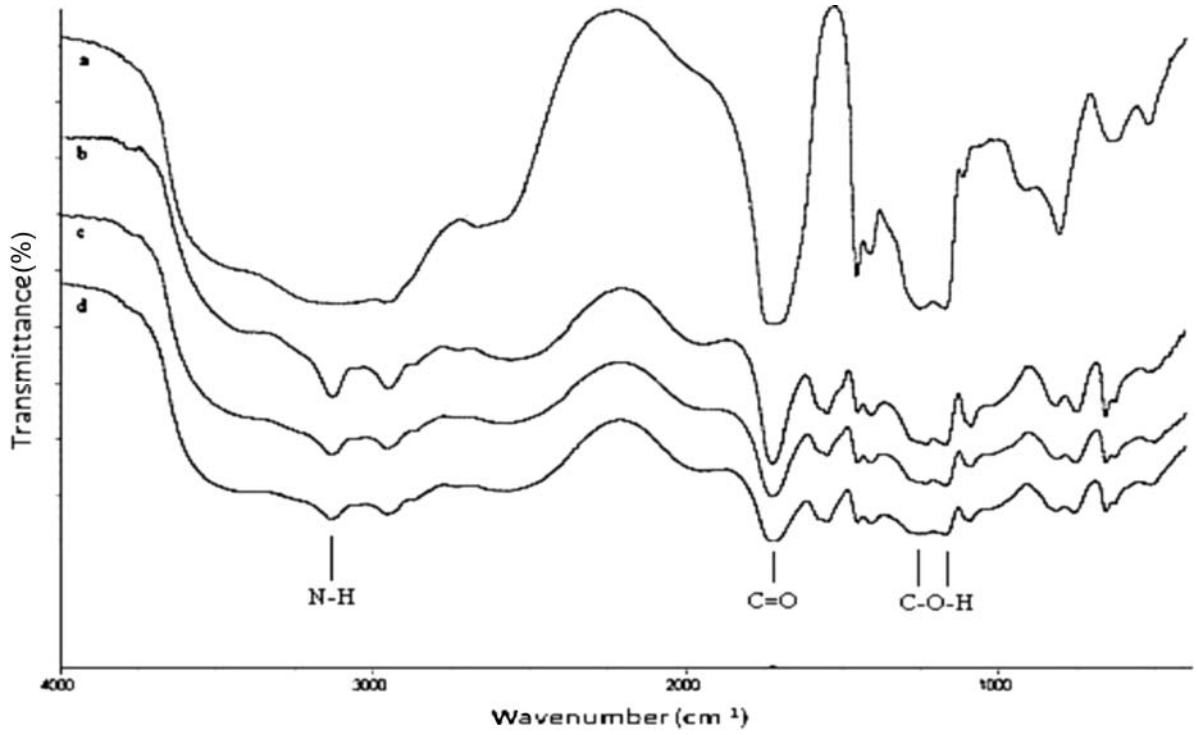

Fig. 2. a) FT-IR of polyvinylphosphonic acid; b) FT-IR of PVPA-PVI (1:1; 1.2; 1.3) polymer complexes.

to behave like a monoprotic acid as given by the titration curve against aqueous NaOH (1). Better complexation in 1:1 ratio can be attributed to this phenomenon.

3.3 Conductivity Measurements

The alternating current (AC) conductivities, σ_{ac} (ω) of the polymers were measured at several temperatures using impedance spectroscopy. Typical AC conductivity σ_{ac} (ω) versus frequency curves are illustrated in Fig. 3 for all PVPA-PVI blends. Frequency dependent and independent regions were observed on the conductivity curves, otherwise known as the characteristic curve for ion conducting polymers. At low frequencies the conductivity increases with frequency up to certain level. After this level conductivity is not affected much by the frequency difference due to electrode polarization at lower frequencies (17). The direct current (DC) conductivities, $\sigma_{dc}(\omega)$ of the samples were derived from plateau regions by linear fitting (Table 2). As a result, conductivity of the PVPA-PVI complex depends both on the molar composition of the polymers and temperature. Moreover, it is confirmed that PVPA-PVI (1:1) polymer blend demonstrates higher proton conductivities than the other compositions especially at temperatures as high as 90°C. The protonation of azole units was confirmed by FT-IR spectroscopy and proton conductivity may occur over the protonated and unprotonated units in the matrix.

Table 1. Elemental analysis results

Complex (PVPA-PVI)	*PVI in the feed (mol%)*	*PVI in the complex (mol%)*
PVPA-PVI(1:1)	50	58
PVPA-PVI(1:2)	66	56
PVPA-PVI(1:3)	75	60

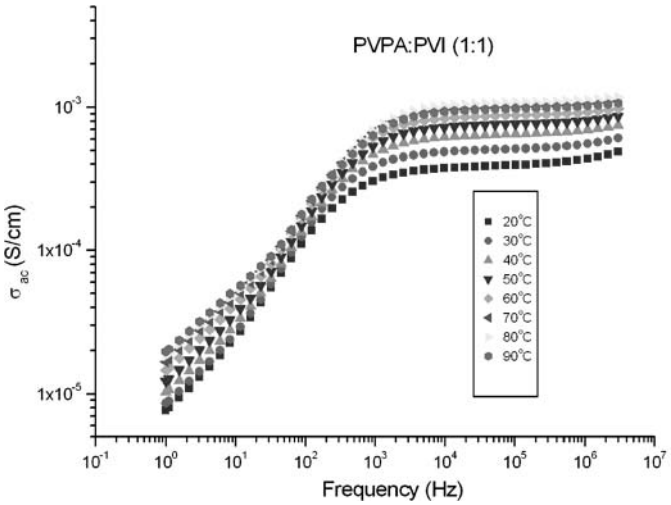

Fig. 3. Frequency dependent AC conductivity curves of a) PVPA-PVI(1:1); b) PVPA-PVI (1:2); c) PVPA-PVI(1:3) at different temperatures.

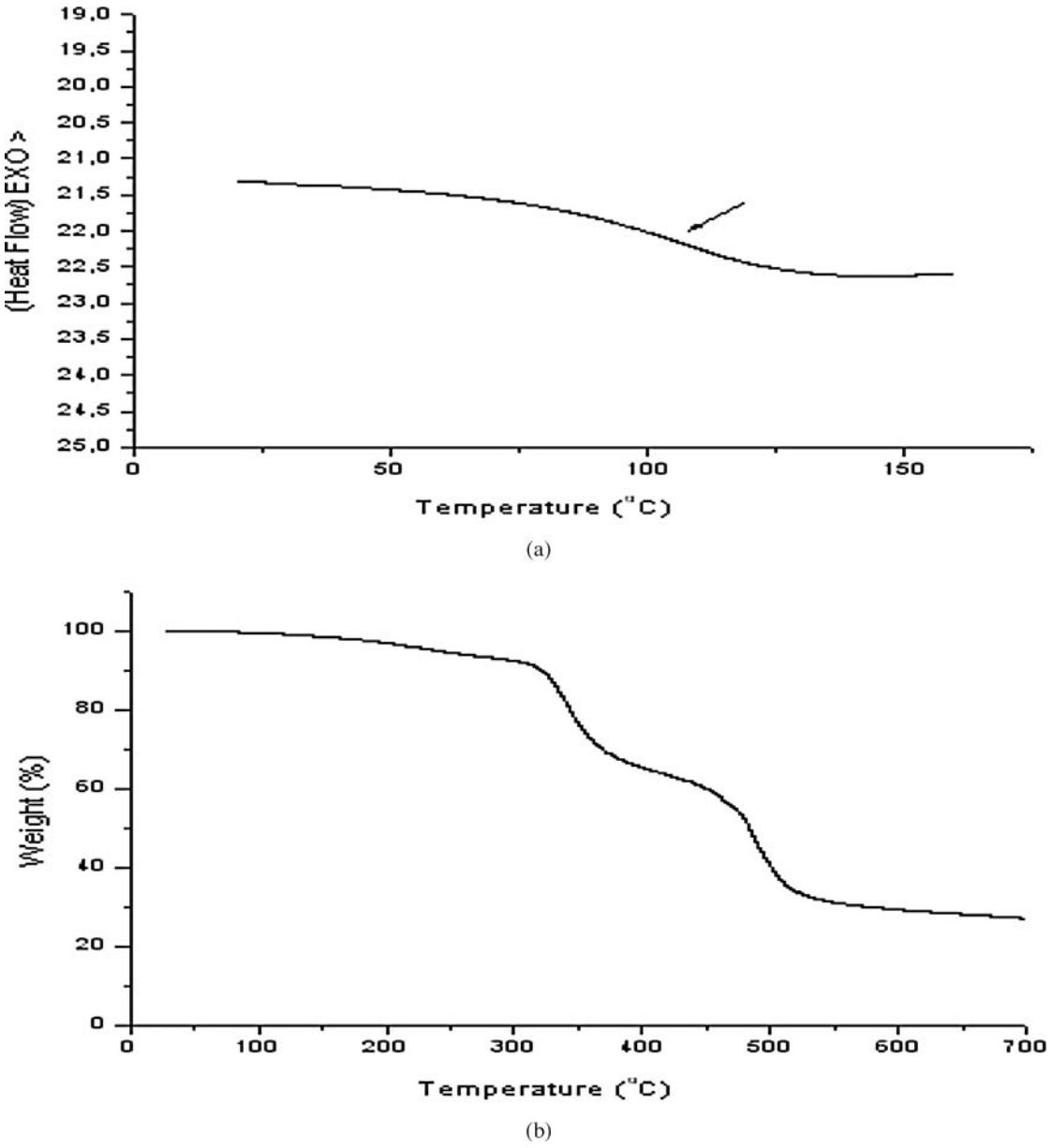

Fig. 4. a) TGA curve of PVPA-PVI(1:1) proton conducting blend with a heating rate of 10°C/min under nitrogen atmosphere b) DSC curve of PVPA-PVI(1:1) proton conducting blend with a heating rate of 10°C/min.

3.4 Differential Scanning Calorimetry Measurements

DSC curves reveal the glass transition temperatures (T_g) of the PVPA-PVI (1:1) blend (Fig. 4a). Prior to measurements samples were loaded into aluminum pans (10–20 mg) and heated to the desired temperature at a rate of 10°C/min under nitrogen flow. The glass transition temperature, T_g of the homopolymer PVPA was reported (7) to have a T_g of -23°C. PVPA–PVI (1:1) blend demonstrates a glass transition temperature at about 100°C. The reason for such a high T_g can be attributed to the restriction of segmental mobility of the polymer chains due to ionic complexation. The presence of a single glass transition is the evidence of the homogeneity of the complex polymer network.

3.5 Thermogravimetry Analysis

Thermogravimetry analysis (TGA) of the PVPA-PVI (1:1) network was made at a heating rate of 10°C/min under nitrogen atmosphere (Fig. 4b). Prior to TGA analysis the samples were dried in a vacuum oven for 5 days. PVPA-PVI (1:1) blend illustrates a slight weight loss between 170 and 300°C. This weight loss was attributed to the elimination of absorbed humidity from these hygroscopic polyelectrolytes (18). Decomposition of complex polymer occurs in two steps in the range 300–500°C as given in Figure 4b. The first weight loss can be correlated with the self condensation of phosphoric acid unit in PVPA above 300°C. The second sharp weight loss occurs due to the decomposition

Table 2. DC Conductivities of the complex polymer electrolytes (RH = 50% for all the materials)

T (°C)	*Blend1:1(S\cm)*	*Blend1:2(S\cm)*	*Blend1:3(S\cm)*
20	3.95E-04	1.06E-05	4.37E-05
30	5.12E-04	1.58E-05	5.89E-05
40	6.61E-04	2.45E-05	9.33E-05
50	7.68E-04	3.72E-05	1.45E-04
60	8.89E-04	5.25E-05	2.24E-04
70	9.12E-04	6.92E-05	3.09E-04
80	1.08E-03	8.32E-05	4.07E-04
90	1.20E-03	9.55E-05	5.13E-04

of the polyelectrolyte at 450°C. This work reveals that complexation of PVPA with PVI prevents a serious weight loss up to 350°C.

3.6 Kinetic Parameters of Immobilized Enzyme

Kinetic parameters V_{max} and K_m were determined (Fig. 5) at constant pH and temperature from Lineweaver–Burk (19) plots ($y = 0.0223x + 2.3859$ $R^2 = 0.9988$). The maximum reaction rate for an enzymatic reaction is given by V_{max}. The Michaelis- Menten constant (K_m) of an enzyme is a measure of the affinity of the enzyme to its substrate. K_m for a particular enzyme is the substrate concentration at which half of the concentration of enzyme molecules is consumed for the formation of enzyme-substrate complex. Table 3 shows that there is a decrease in K_m values compared to that of the free enzyme. The low K_m value indicates higher enzyme–substrate affinity. The substantial decrease in K_m value leads to the tendency of enzyme to bind its substrate more strictly than the free enzyme does, hence, enzyme substrate complex stays together for a long time that makes the enzymatic reaction rate rather slower. In this network, K_m and V_{max} decrease due to easier enzyme-substrate interaction.

3.7 Temperature and pH influence

Enzyme immobilization at optimum temperature and pH is crucial for biosensor applications. The effect of temperature on the enzyme activity is shown in Figure 6. Although free invertase completely lost its activity (20) at 50°C, immobilized invertase in PVPA/PVI network keeps its activity up to temperatures as high as 70°C with a maximum at 60°C. Moreover, this matrix shows adequate activity at low temperatures. For pH optimization experiments, a pH range from 5 to 9 was investigated while the temperature and concentration of the medium were kept constant (Fig. 7). In previous works, the maximum activity of free invertase was observed at pH 5 (19). Invertase entrapped in the PVPA-PVI matrix showed a maximum activity at pH 7. Invertase immobilized PVPA-PVI network reveals appreciable activities in a wider pH range compared to free invertase. Moreover, although free invertase does not show activity at high pH values, with present entrapment method invertase demonstrates efficient activity up to pH 8.

Table 3. Kinetic parameters of the immobilized enzyme

	V_{max}	K_m (mM)
Free Invertase	82.3 μmol/min.mL	24.3
PVPA-PVI Complex	0.497 μmol/min.mg complex	6.8

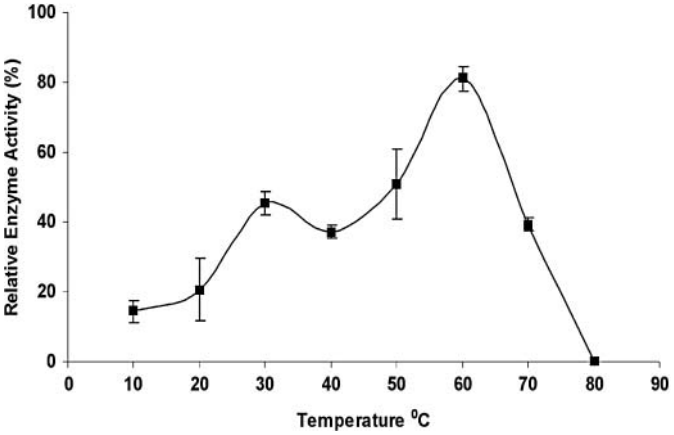

Fig. 6. Optimum temperature for the enzyme biosensor.

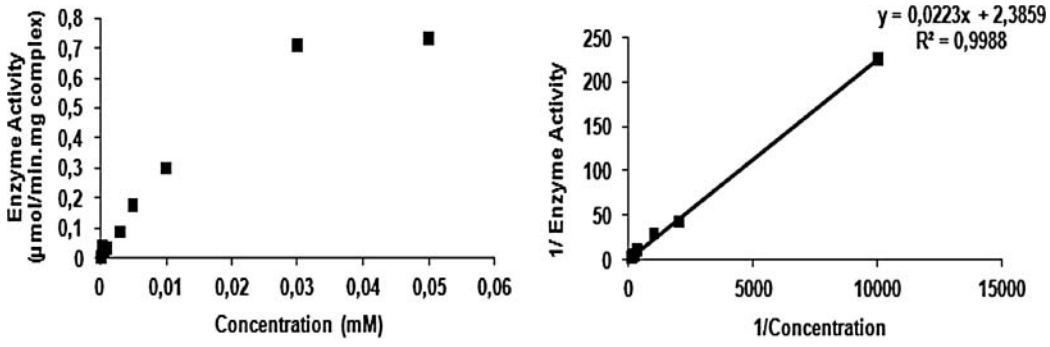

Fig. 5. Lineweaver Burk Plot for the immobilized enzyme.

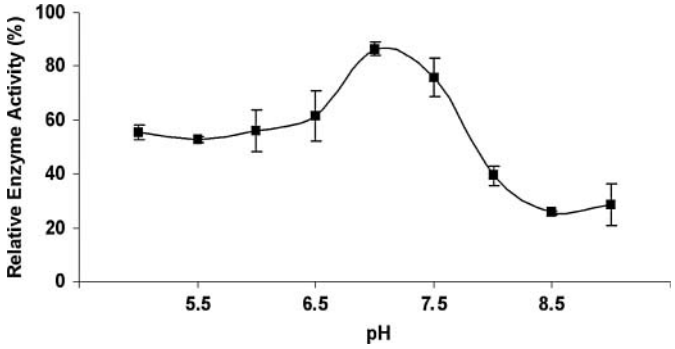

Fig. 7. Optimum pH for the enzyme biosensor.

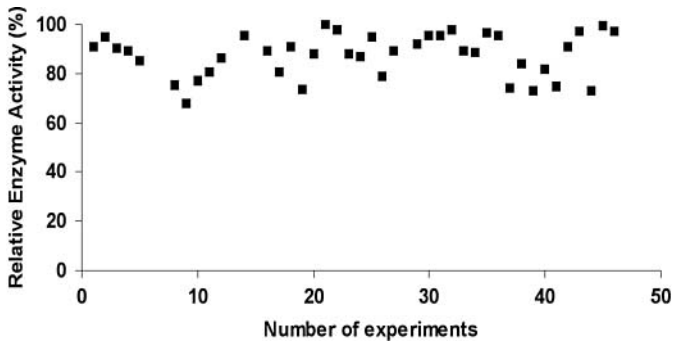

Fig. 8. Operational stability of the enzyme biosensor.

3.8 Operational, storage and thermal stability

Enzymes can easily be denaturated by losing their catalytic activity thus, stability in repetitive use and storage is essential. Free enzymes cannot be recovered from the solution thus; operational experiments are only valid for immobilized enzyme matrices. Proton conducting matrix used for immobilization of invertase helps to keep nearly 30 % initial activity of enzyme after 25 repetitive usages (Fig. 8). The further activity loss of the enzyme was not observed even after several experiments. For storage stability experiments the activity of invertase immobilized PVPA-PVI biosensor

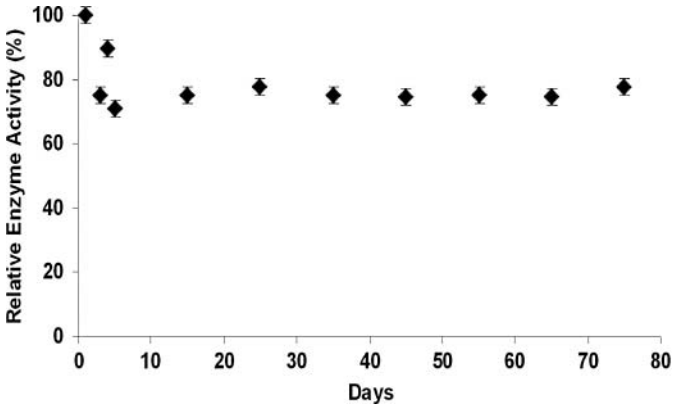

Fig. 9. Storage stability of the enzyme biosensor.

Table 4. Thermal stabilities

Temperature (°C)	*Decrease in Activity (%)*
30	40
40	34
50	29
60	27

was measured for every 10 days within consecutive 75 days. This enzyme-network system conserves substantial activity within 75 days (Fig. 9). The enzymatic activity stays almost constant during such a long storage period although free invertase loses its activity within only 8 days (21). Comparing with the results of previous works (3,15), PVPA-PVI complex shows superior stability over other matrices. Thermal stability profile of the immobilized invertase was determined at different temperatures (30, 40, 50°C). As shown in Table 4, immobilized invertase was significantly more resistant to heat treatment at temperatures higher than 40°C, in comparison with activity values at lower temperatures. An operational stability experiment was performed at optimum temperature and pH with 25 repetitive usages. Upon immobilization of enzyme in network, thermal stability was more pronounced at higher temperatures.

4 Conclusions

Invertase is a well known enzyme for the immobilization purposes in biosensor designing. In this work immobilization of invertase in a novel polymer network matrix based on poly(vinylphosphonic acid) (PVPA) and poly(1-vinylimidazole) (PVI) was achieved. The polymer matrix was characterized by FT-IR, elemental analysis, proton conducting, impedance spectroscopy, DSC and TGA analyses. IR spectra and impedance measurements show that proton conductivity was achieved by the transfer of hydrogen between proton donor and acceptor species. Maximum conductivity was determined as 1.20×10^{-3} at 90°C for PVPA-PVI (1:1). Immobilization of invertase was achieved via physical entrapment in a novel proton conducting polymer electrolyte. Characterization and optimization experiments for the immobilized invertase were performed. This study reveals that immobilization of invertase into the complex polymer electrolyte matrix has been successfully achieved. With the characterization of the complex and storage stability experiments, it is possible to state that the most stable and highly proton conducting polymer network was synthesized for biosensor applications.

Acknowledgments

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