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Immobilization of invertase in conducting polymer matrices

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This paper reports a novel approach in the electrode immobilization of an enzyme, invertase, by electrochemical polymerization of pyrrole in the presence of enzyme. The polypyrrole/invertase and polyamide/polypyrrole/invertase electrodes were constructed by the entrapment of enzyme in conducting matrices during electrochemical polymerization of pyrrole. This study involves the preparation and characterization of polypyrrole/invertase and polyamide/polypyrrole/invertase electrodes under conditions compatible with the enzyme. It demonstrates the effects of pH and temperature on the properties of enzyme electrode. Enzyme leakage tests were carried out during reuse number studies. The preparation of enzyme electrodes was done in two different electrolyte/solvent systems. The enzyme serves as a sucrose electrode and retains its activity for several months.

Keywords: Immobilization, conducting polymer matrices, invertase, polymerization

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Since polypyrrole was first electrochemically synthesized by Diaz *et al.*[1] there has been great interest in the study of polypyrrole and its potential technological applications as a secondary battery[2], protective coating[3] and electrochromic devices[4] due to its high conductivity, thermal stability and environmental stability. In addition to these advantages, its stability in aqueous solution makes polypyrrole attractive in biomedical applications. Various studies in this area using the property of the biomolecule release system have been done[5–7].

Immobilization of enzyme can be achieved by physical entrapment in an inert matrix and chemical immobilization to an inert matrix with bifunctional reagents, covalent attachment onto an activated electrode surface and covalent attachment of chemically modified enzyme with an electron relay to an electrode surface. One alternative approach, the entrapment of enzymes in conducting polymer matrices during electrochemical polymerization, is attracting great interest, because it is simple, speedy, reliable and inexpensive, and the immobilization procedure involves only the application of a suitable potential on an electrode in appropriate aqueous solutions of monomers and enzymes. Recently, polypyrrole has been used as a conducting matrix for fixing enzymes. The resulting films may be useful as biosensor elements. The first publication dates back to 1986 and deals with the immobilization of glucose oxidase[8,9]. Subsequently, many reports have described the development of glucose sensors with immobilized glucose oxidase[10–13].

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Close contact of enzymes with conducting polymers shows improvement in the efficiency of electrochemical signal from enzyme to electrode. However, the usefulness of immobilization of enzyme in electrodes depends on factors such as the immobilization method, the chemical and physical conditions, like pH and temperature, contaminants, the thickness and the stability of the membrane to entrapment, the activity and stability of the enzyme when immobilized, the stability of the sensor, the response time and the storage conditions. Although the immobilization by electrochemical polymerization appears straightforward, the formation of chemical bonds between the electrode surface and the enzyme may cause deformation of the enzyme's skeleton and a decrease in the specificity of enzymatic reactions and activity. To avoid degradation of enzyme and hence a decrease in its activity, the conditions of immobilization must be kept in the range where the stability of the enzyme is not endangered.

Polypyrrole films are permeable to ions and neutral species. They can be switched between conducting and insulating states. Oxidized (conducting) polypyrrole can be regarded as a porous material, with an average pore size of 5–10 nm. On the basis of these considerations, polypyrrole can be used as an electron and mass transport medium for enzymes and the reaction products can be detected amperometrically.

There are several advantages of enzyme electrodes, such as the easy analyte determination in complex mixtures, the use of small sample volumes and easy recovery of enzyme for repeated use. Immobilization of an enzyme on an electrode allows us not only to develop various sensors to detect the components of

biochemical systems in medical applications, but also to employ relatively simple electrochemical methods to study the reactions participating in biochemical regulation mechanisms *in vitro* as well as *in vivo*.

Invertase is an important enzyme in biological systems, where it plays a catalytic role in the conversion of sucrose to glucose and fructose. Immobilized invertase can be used in the food industry, since it prevents the production of coloured byproducts by acidic hydrolysis processes and crystallization when it is used in the hydrolysis of sucrose to yield sugar mixtures.

Immobilization of invertase on corn grits[14], gelatin[15], carbohydrate moieties[16] and polyelectrolytes[17] has already been achieved, while its entrapment in conducting polymer matrices has been limited[18]. In this study we examined the immobilization of invertase on polypyrrole (PPy) and polyamide/polypyrrole (PA/PPy) matrices during electrochemical polymerization of pyrrole in the presence of invertase. The preparation of the PA/PPy electrode was discussed in detail in an earlier study[19]. For immobilized electrodes, optimum parameters such as pH, temperature, sucrose concentration and kinetic parameters were examined by spectroscopic means. Shelf life and operational stability of the immobilized enzymes were given.

MATERIALS AND METHODS

Apparatus

A Potentioscan Wenking POS-73 model potentiostat and a Shimadzu UV-1601 model spectrophotometer were used.

Materials

Invertase (β-fructofuranosidase), type V (EC No. 3.2.1.26) was purchased from Sigma and $NaClO_4$, pyrrole and sodium dodecyl sulphate (SDS) from Aldrich. The pyrrole was distilled before use.

Immobilization of invertase in conducting polymer matrices

Electrochemical experiments were performed in a conventional three-electrode electrochemical cell using platinum (Pt) electrodes (1.5 cm^2 each) as the working and counter electrodes, and silver/silver ion as a reference electrode at room temperature. Immobilization of invertase was carried out by electrochemical polymerization of pyrrole in 50 ml of distilled water containing 0.4 mg ml^{-1} $NaClO_4$, 0.01 M pyrrole and 0.4 mg ml^{-1} invertase on either bare or polyamide-coated Pt electrodes at 0.7 V. Another system used for the immobilization of invertase contained 0.01 M pyrrole, 0.4 mg ml^{-1} invertase and 0.4 mg ml^{-1} SDS in acetate buffer (50 mM, pH 5). Oxidative deposition of pyrrole on bare or polyamide-coated Pt electrodes was carried out at 1 V for this system.

Solutions in the cell were purged with nitrogen for 5–10 min before each polymerization and a blanket of nitrogen was supplied during the experiment.

After polymerizations, the electrodes were kept in acetate buffer (50 mM, pH 5) for a certain time interval. This solution was examined for enzyme activity due to the leakage of any unbound enzyme. The procedure was repeated by placing the electrodes in another solution until no activity was observed. The electrodes were kept at 4 °C in acetate buffer when not in use.

Determination of invertase activity

The immobilized and free invertase activities were determined using the Nelson method. For free enzyme activity, 0.08 ml of 50 mM acetate buffer (pH 5) and the enzyme sample (0.1 ml) were placed in a test tube. Following a preincubation period (2 min at 25 °C), 0.1 ml of substrate solution (0.3 mol dm^{-3} sucrose) was added into the samples and the incubation was continued for exactly 3 min. The tubes were then removed from the water bath and 1 ml of Nelson's reagent was added to terminate the reaction. The tubes were then placed in a boiling water bath for 20 min, cooled and 1 ml arsenomolybdate reagent was added to each sample tube and mixed well by vortexing. Finally, 7 ml of distilled water was added to each test tube. The optical density of the medium was measured at 540 nm using a Shimadzu UV–visible spectrophotometer.

Determination of immobilized invertase activity

To test the enzyme activity, invertase electrodes were put into a test tube containing 3.6 ml acetate buffer corresponding to each electrode's optimum pH and incubated for 2 min at 25 °C. Sucrose solution (0.4 ml, 0.3 M) was then added. Following an incubation period (3 min at 25 °C) the electrodes were removed and 1 ml aliquots were drawn and added to 1-ml Nelson's reagent. The remainder of the procedure was as for free enzyme activity measurements.

For blank measurements (to eliminate the interference that may come from the PPy/Pt and PA/PPy/Pt electrodes), the same procedure in the absence of enzyme was carried out.

Determination of optimum pH

To evaluate the pH stability, the electrodes were preincubated with the corresponding buffer (3.6 ml acetate buffer in the pH range 4.2–5.8, phosphate buffer in the range 6.0–7.5) for 2 min at 25 °C and 0.4 ml of 0.3 M sucrose solution was added. Following an incubation period (1 min) the electrodes were removed and 1-ml aliquots were drawn and added to 1 ml Nelson's reagent. The rest of the procedure was as for free enzyme activity measurements.

Determination of optimum temperature

The electrodes were placed in 3.6 ml acetate buffer (at a determined pH for each electrode) and preincubated for 2 min in the temperature range 10–90 °C. After preincubation, 0.4 ml sucrose solution was added and allowed to stand for 1 min. Following the incubation, 1-ml aliquots were drawn and added to 1 ml Nelson's reagent. The rest of the procedure was as for free enzyme activity measurements.

RESULTS AND DISCUSSION

The electrochemical polymerization of pyrrole in the presence of invertase yielded a black film on the

electrode surface. The thickness of the films deposited was 20 μm. It was found that a concentration of 0.01 M pyrrole in the presence of 0.4 mg ml^{-1} invertase in 0.4 mg ml^{-1} $NaClO_4$ were the optimal parameters for the fabrication of electrode in the $NaClO_4$/water system. Visual examination of the surface of the electrodes revealed a homogeneously covered surface with black films. The concentration of invertase where the polymerization of pyrrole was carried out was not chosen arbitrarily. Above 0.4 mg ml^{-1} concentration, the amount of invertase incorporated into the conducting polymer matrices was not affected.

In order to check the activity of the polyamide-coated electrode, it was placed in an electrolysis cell in the absence of pyrrole. It was electrolysed under the same conditions as when pyrrole was present in the medium. The results showed that the entrapment of enzyme was not realized on the polyamide surface.

The activity of free enzyme (0.4 mg ml^{-1}) in the presence of both SDS and $NaClO_4$ was checked. When the concentration of both electrolytes in polymerization media was 0.4 mg ml^{-1}, the activity of enzyme was found to be the same as that of the free enzyme (without the electrolyte in the medium). It was observed that none of the electrolytes caused deactivation of the enzyme within the concentration limits used during electrolyses. When the concentration of electrolytes was increased to 40 mg ml^{-1}, deactivation of enzyme was observed. The activities of enzyme in two different media are given in *Table 1* as the relative activity of enzyme at different electrolyte concentrations. The activity in $NaClO_4$ solution was 90% of that of the free enzyme, whereas SDS solution caused a 37% decrease in activity.

Enzymes are very fragile molecules when removed from their natural medium. A change in temperature, pH or ionic media may cause denaturation. During electrochemical polymerization of pyrrole, protons are released and this leads to a decrease in pH. For this reason it is essential to use a buffer solution during electrolyses to avoid denaturation of the enzyme. Invertase has an isoelectric point of 4.5 and is therefore negatively charged at the pH (pH 5) used for the electrodeposition, and could be incorporated into the polymer as a counter ion of the PPy during electrochemical polymerization. However, although the entrapment of negatively charged enzymes acts as counter anions[20], it is more likely that the entrapment is only due to an enclosure of the enzymes present in the vicinity of the electrode surface. The enzyme electrodes prepared in SDS/50 mM acetate buffer/electrolyte/solvent systems showed better responses compared to ones prepared in the $NaClO_4$/water system.

The immobilization of invertase onto conducting polymer matrices, especially the one prepared in the SDS/acetate buffer (50 mM, pH 5) system, yielded highly active and stable enzyme (*Table 2*). It was thought that the decrease in pH caused the denaturation of the enzyme during electrolysis. The same set of experiments using $NaClO_4$ was carried out in acetate buffer (50 mM, pH 5). For this specific case it takes about four times as long to obtain one fifth of the amount immobilized in the SDS/acetate buffer (50 mM, pH 5) system. In addition, the films obtained in this way were not smooth and homogeneous.

The conductivities of the enzyme-entrapped films lie in the range of 0.001–0.01 S cm^{-1}. These low conductivities, compared to our previous studies, were attributed to low electrolyte concentration where the immobilizations were carried out[19].

Table 1 Relative activities of enzyme at different concentrations with respect to the free enzyme

	0.4 mg ml^{-1}	40 mg ml^{-1}
$NaClO_4$	100	90.1
SDS	100	63.18

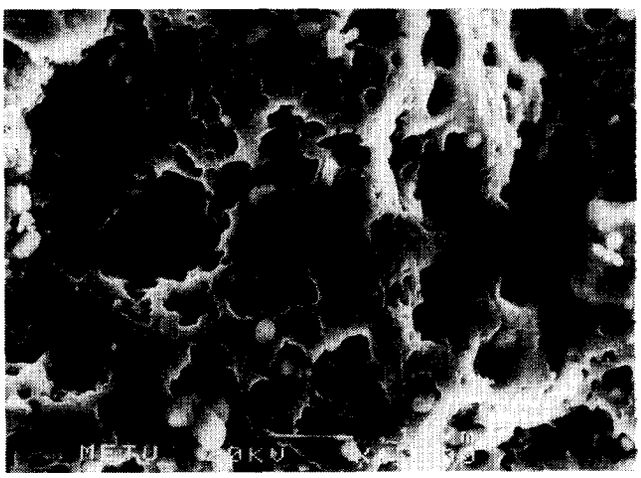
Table 2 Relative activities (μmol per min per electrode) of enzyme electrodes prepared in different media

	ClO_4^-	SDS
PPy	0.107	0.733
PA/PPy	0.0735	0.325

Morphology of enzyme-entrapped films

Scanning electron micrographs of PPy/ClO_4^-, PA/PPy/ClO_4^-, PPy/SDS and PA/PPy/SDS are given in *Figures 1–4*, where the films were washed before

a



b

Figure 1 Scanning electron micrographs of PPy/ClO_4^- electrode side: **a**, without enzyme; **b**, with enzyme entrapped in the matrix.

a

b

Figure 2 Scanning electron micrographs of PPy/SDS solution side: **a,** without enzyme; **b,** with enzyme entrapped in the matrix.

analysis in order not to see the enzyme which physically adsorbed on the surface. The examination of the electrode sides of PPy/ClO_4^- films in the presence of enzyme (*Figure 1b*) revealed that an obvious morphological change occurred. The additional structures that were seen can be attributed to enzyme clusters. The solution sides of PPy/SDS films revealed cauliflower-like structures together with enzyme clusters (*Figure 2b*). Significant changes were also observed for the solution side of $PA/PPy/ClO_4^-$. As can be seen in *Figure 3*, the appearance of cauliflower-like structures which were easily seen in the absence of enzyme decreases and the surface becomes stiffer. An obvious difference was also observed on the solution side of the enzyme-entrapped PA/PPy/SDS film compared to that without enzyme (*Figure 4*). The presence of SDS considerably improves the homogeneity and makes the films more flexible.

Effect of pH on the enzyme electrode response

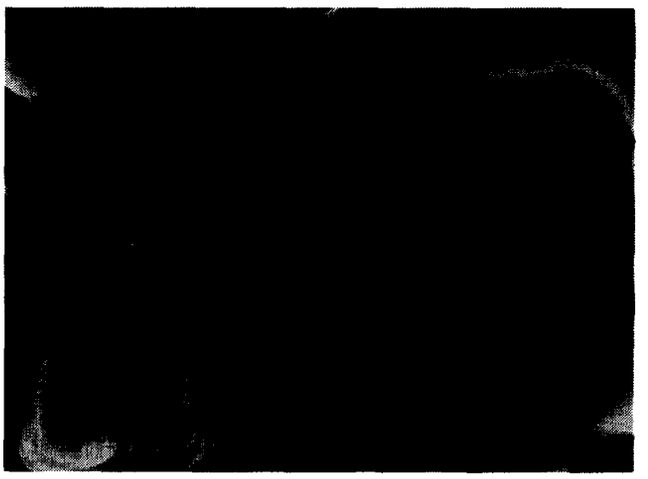
In order to find the optimum pH for the enzyme, acetate and phosphate buffers were utilized in the pH range 4.2–7.5. The optimum pH of the enzyme was only slightly affected by the immobilization process. For the soluble enzyme and PA/PPy/SDS electrode, the optimum pH was 5.0 (*Figure 5*). The invertase immobilized onto PA/PPy electrodes prepared in $NaClO_4$ caused a shift of 0.6 units into the acidic range of the optimum pH. This value for PPy electrodes prepared both in SDS and $NaClO_4$ was 5.6.

Effect of temperature on the enzyme electrode response

The effect of temperature was determined using a 0.03 M sucrose solution at optimum values for each electrode from 10 to 90 °C. The optimum temperature for free enzyme was 60 °C. The optimum temperature for PA/PPy matrix prepared either in SDS or $NaClO_4$ was 50 °C. The enzyme entrapped within polypyrrole matrices showed higher stability against temperature and the optimum temperature here was 70 °C for both SDS and $NaClO_4$ electrolytes (*Figure 6*).

Shelf life and operational stability of the enzyme electrode

Important parameters when considering immobilized enzymes are the lifetime, durability and storage stability of the system. The electrodes were kept at 49 °C in acetate buffer (50 mM, pH 5) when not in use. PPy/ClO_4^- and $PA/PPy/ClO_4^-$ electrodes retained their initial activities even after 2 months. Results have



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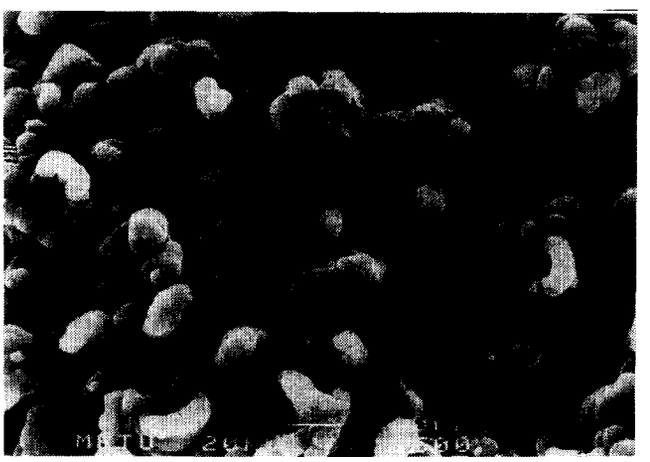

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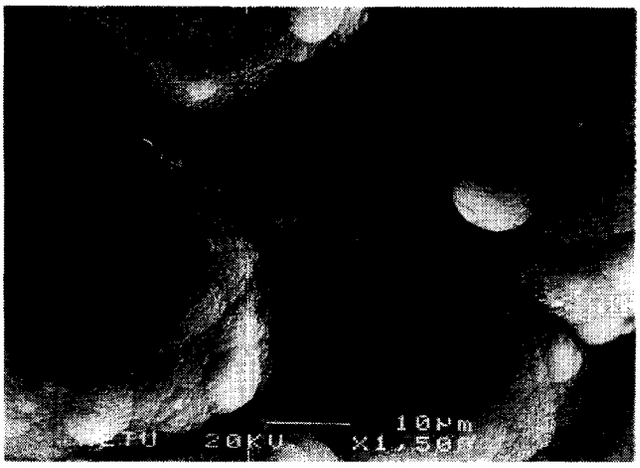
Figure 3 Scanning electron micrographs of $PA/PPy/ClO_4^-$ solution side: **a,** without enzyme; **b,** with enzyme entrapped in the matrix.



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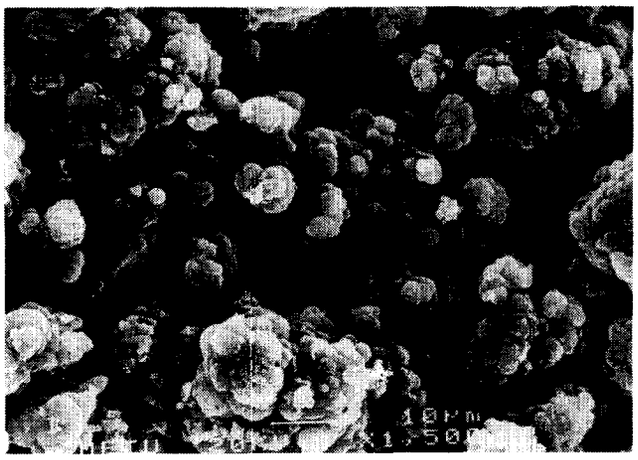

b

Figure 4 Scanning electron micrographs of PA/PPy/SDS solution side: **a**, without enzyme; **b**, with enzyme entrapped in the matrix.

shown that 72% of the initial activity was maintained for the PPy/SDS electrode, whereas PA/PPy/SDS retains 55% of initial activity within the same period.

The operational stability was studied on 12 assays as shown in *Figure 7*. The response of the PPy/SDS electrode showed a maximum value within a few days and relative activity decreased to 56% after day 18. The PA/PPy/SDS electrode shows better responses compared to PPy/SDS. It retained 73% of its initial activity. The PPy/ClO_4^- and PA/PPy/ClO_4^- electrodes lost 17% and 12% of their initial activities,

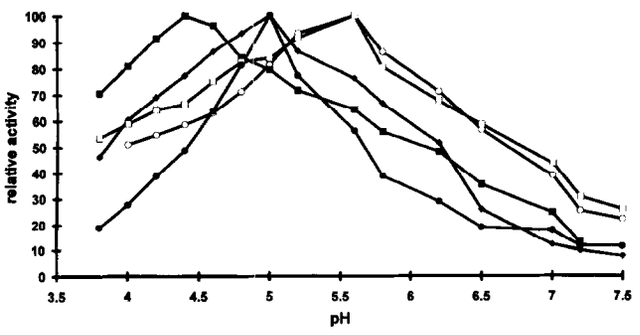

Figure 5 Effect of pH on the activity of free and immobilized invertase. ◆, Free enzyme; □, PPy/ClO_4^-; ■, PPy/PA/ClO_4^-; ○, PPy/SDS; ●, PPy/PA/SDS.

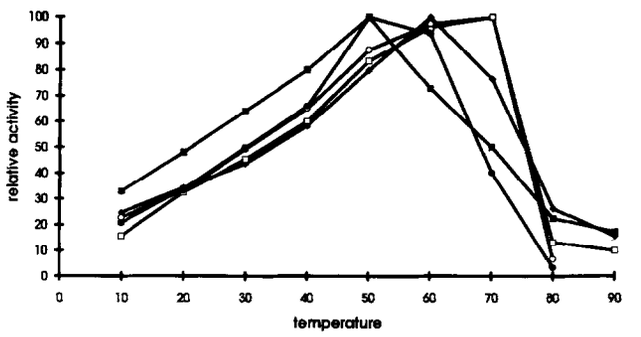

Figure 6 Effect of temperature on the activity of free and immobilized invertase. ◆, Free enzyme; □, PPy/ClO_4^-; ■, PPy/PA/ClO_4^-; ○, PPy/SDS; ●, PPy/PA/SDS.

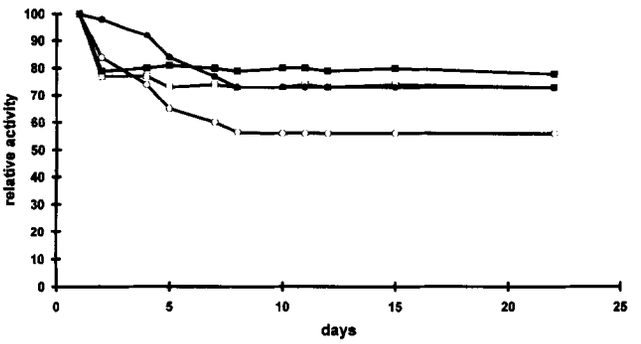

Figure 7 Effect of repeated use on the activity of immobilized invertase. □, PPy/ClO_4^-; ■, PPy/PA/ClO_4^-; ○, PPy/SDS; ●, PPy/PA/SDS.

respectively. The activities remained constant on the subsequent days (*Figure 7*). The activities of electrodes were checked at the end of 2 months and no loss was observed. The initial loss of activity can be attributed to structural changes in the enzyme during the immobilization process.

Kinetic studies of free and immobilized invertase

The Lineweaver–Burk plots of $1/V$ vs $1/C_{sucrose}$ of the electrodes that were prepared under optimum conditions were drawn. The V_{max} and Michaelis–Menten constant, K_m, were calculated from the intercept and the slope of the straight line. Kinetic constants for the hydrolysis of sucrose by free and immobilized invertase are given in *Table 3*. In all cases, the increase in the K_m values was attributed to electrostatic interaction between the carrier and the substrate and diffusional effects, i.e. internal mass transfer resistance arises when enzymes are entrapped in polymers. The formation of enzyme–substrate complex is more difficult with the immobilized invertase due to the porous structure of the conducting polymer, which causes the substrate to pass with

Table 3 Kinetic constants for sucrose hydrolysis by free invertase and immobilized invertase

	K_m (mM)	V_{max} (μmol min^{-1})
Free invertase	30.2	98
In PPy/ClO_4^- matrix	33.9	1.84
In PA/PPy/ClO_4^- matrix	94.5	1.81
In PPy/SDS matrix	63.0	3.70
In PA/PPy/SDS matrix	50.0	3.07

difficulty through the pores. The differences in the K_m values for each electrode may be attributed to the differences in porosity of each polymer film. Possible decrease of reaction rate is due to the increased concentration of product within the pores that may not be removed after successive measurements.

CONCLUSIONS

This study shows that conducting polymers can be used successfully for the immobilization of invertase. The entrapment of invertase onto conducting polymer matrices provides some advantages such as ease of incorporation of invertase onto conducting polymer matrices and fast response to substrate. The electrochemical polymerization of pyrrole is an inexpensive polymerization procedure which readily yields polymer films. Incorporation of SDS as a counter ion in the polymer matrix yielded five- to six-fold increases in the enzyme activity. The excellent mechanical strength of polymers with immobilized enzymes eases handling during electrode fabrication.

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