Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioelectrochemistry 59 (2003) 29–33
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Immobilization of invertase in conducting copolymers of 3-methylthienyl methacrylate

A. Cirpan[a], S. Alkan[a], L. Toppare[a,*], Y. Hepuzer[b], Y. Yagci[b]

[a] *Department of Chemistry, Middle East Technical University, 06531 Ankara, Turkey*
[b] *Department of Chemistry, Istanbul Technical University, 80626 Istanbul, Turkey*

Received 20 August 2002; accepted 4 October 2002

Abstract

Immobilization of invertase in conducting copolymer matrices of 3-methylthienyl methacrylate with pyrrole and thiophene was achieved by constant potential electrolysis using sodium dodecyl sulfate (SDS) as the supporting electrolyte. Polythiophene (PTh) was also used in entrapment process for comparison. Kinetic parameters, Michaelis–Menten constant, K_m, and the maximum reaction rate, V_{max}, were investigated. Operational stability and temperature optimization of the enzyme electrodes were also examined.

Keywords: Immobilization; Invertase; Conducting copolymer; Electropolymerization; Polythiophene; Polypyrrole

1. Introduction

An enzyme is a substance that acts as a catalyst in living organisms, regulating the rate at which chemical reactions proceed without itself being altered in the process. Enzymes also have valuable industrial and medical applications. The fermenting of wine, leavening of bread, curdling of cheese, and brewing of beer have been practiced from earliest times, but not until the 19th century were these reactions understood to be the result of the catalytic activity of enzymes. Immobilization of enzyme is undertaken either for the purpose of basic research or for use in technical processes of commercial interests. Immobilization means that enzyme, while retaining their catalytic activity, is confined within a certain space or is bound to solid carriers or to one another. Immobilization of enzyme has several advantages. These are: (i) the stability of the enzyme by protecting the active material from deactivation; (ii) repeated use; (iii) significant reduction in the operation cost; (iv) easy separation and recovery of the enzyme. Advantages of immobilization of enzymes in conducting polymer by electropolymerization are an easy one-step procedure, accurate control of the polymer thickness via the electrical charge passed during the film formation process, localization of the electrochemical reaction exclusively on the electrode surface allowing precise modification of micro electrodes and surfaces of complex geometry and the possibility to build up multilayer structures.

Invertase or β-fructofuranosidase (EC No.3.2.1.26) catalyses the hydrolysis of sucrose to glucose and fructose which is known as invert sugar. The immobilization of invertase on polyethylene [1], polyaniline [2] corn grifts [3], gelatin [4], carbohydrate moieties [5], polyelectrolytes [6], porous cellulose beads [7], diazonium salt of 4-aminobenzoylcellulose [8] and poly (ethylene-vinylalcohol) [9] has been studied. The invertase enzyme has been immobilized into conducting copolymers by electropolymerization [10–12].

In this study, immobilization of invertase via electropolymerization within poly (3-methylthienyl methacrylate) (PMTM), polythiophene (PTh) and PMTM/PTh copolymer matrices was investigated. The synthesis and characterization of conducting copolymers of PMTM/PPy and PMTM/PTh were reported in an earlier study [13]. Kinetic parameters (reaction rate, V_{max}, and Michaelis Menten constant, K_m) of enzyme electrodes were determined. Temperature optima curves and operational stabilities of immobilized invertase were examined. It was found that PPy and PTh were better matrices for invertase enzyme.

* Corresponding author. Tel.: +90-312-2103251; fax: +90-312-2101280.
E-mail address: toppare@metu.edu.tr (L. Toppare).

1567-5394/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1567-5394(02)00186-X

2. Experimental

2.1. Materials

Invertase (β-fructofuranosidase) type V (EC No.3.2.1.26) was purchased from Sigma and used as received without further purification. Pyrrole (Sigma) and thiophene (Sigma) were distilled before use and stored at 4 °C. Sodium dodecyl sulfate (SDS) (Sigma) and tetrabutylammonium tetrafluoroborate (TBAFB) were used as received. For the preparation of Nelson reagent, sodium carbonate, sodium potassium tartarate, sodium bicarbonate, sodium sulfate, copper sulfate and for the preparation of arsenomolibdate reagent, ammonium heptamolibdate, sodium hydrogen arsenate were used without any purification.

2.2. Synthesis of copolymers

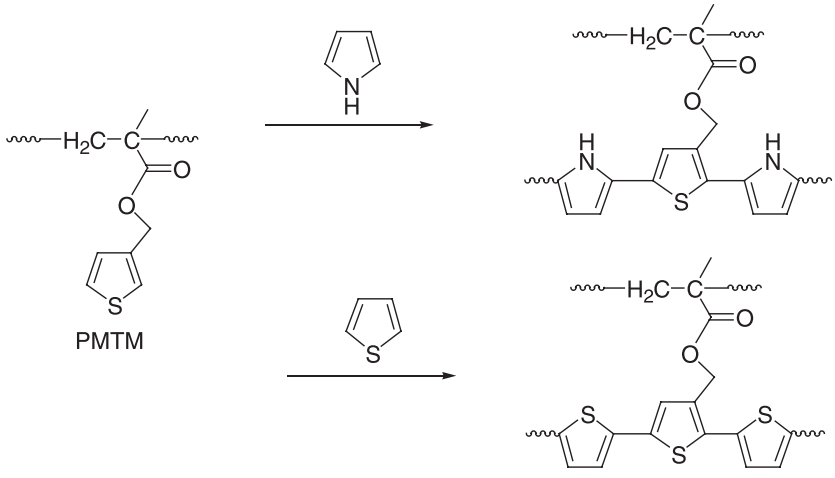
Electropolymerization of PMTM was achieved in the presence of pyrrole and thiophene by constant potential electrolysis (Scheme 1).

2.3. Preparation of enzyme electrodes: 1-PPy/SDS, 2-PMTM/PPy/SDS, 3-(PMTM/PPy)/SDS, 4-(PMTM/PTh)/PPy/SDS and 5-(PTh)/PPy/SDS

Immobilization of invertase was accomplished by electropolymerization of pyrrole either on bare or previously PMTM coated platinum (Pt) electrode (enzyme electrodes 1 and 2). The electrolyses were carried out at +1.0 V in buffer that contains invertase, sodium dodecyl sulfate (SDS) and pyrrole. For the preparation of enzyme electrode 3, PMTM/PPy copolymer was prepared in acetonitrile–TBAFB solvent–electrolyte couple at +1.0 V for 60 min; then it was reduced in the same system at +0.1 V for 30 min. The reduced copolymer film was immediately moved into the buffer containing invertase and SDS, and oxidized at +1.0 V for 100 min. As to the enzyme electrodes 4 and 5 (Table 1), PMTM/PTh copolymer and PTh films were again prepared in acetonitrile–TBAFB, solvent–electrolyte pair at +1.9 V for 30 min, since thiophene is not soluble in buffer. Then, freshly prepared films were transferred into another cell containing 15 ml acetate buffer, 0.6 mg/ml SDS, 0.6 mg/ml invertase and 0.01 M pyrrole. Electrolyses were performed at +1.0 V for 100 min. Immobilization was performed in a one compartment cell, consisting of Pt working and counter electrodes and a Ag/Ag^{+} reference electrode. A potentioscan Wenking POS-73 model potentiostat was used for electropolymerization. All electrolyses were carried out at room temperature and under nitrogen atmosphere. After immobilization was achieved, electrodes were removed and washed several times with distilled water to remove the supporting electrolyte. Next, the electrode was placed in acetate buffer for 10 min and solution was examined for the enzyme activity due to unbound enzyme. This procedure was repeated for several times with the acetate buffer until no activity was observed. Electrodes were stored in acetate buffer at 4 °C when not in use.

2.4. Activity determination of invertase

The activities of immobilized invertase activity were determined by using Nelson's method [14]. Different concentration of sucrose solutions prepared in acetate buffer (pH 7) were placed in test tubes and moved to water bath at 25 °C for 10 min. Enzyme electrode (EE) was immersed in the test tubes and shaken in the water bath for 2 min. The electrodes were removed; 1 ml aliquots were drawn and added to 1 ml Nelson's reagent to terminate the reaction. The tubes were then placed in boiling water bath for 20 min, cooled and 1 ml arsenomolybdate was added. Finally, 7 ml of distilled water was added to each test tube and mixed well by vortexing. After mixing, absorbances for the blank and



Scheme 1. Electrochemical synthesis route for copolymerization.

Table 1
Invertase activities in PMTM/PPy/SDS and (PMTM/PPy)/SDS polymer matrices

Polymer matrix	Activity (μmol min^{-1} electrode^{-1})
PMTM/PPy/SDS	2.053
(PMTM/PPy)/SDS	0.188

the solutions were determined at 540 nm with a double beam spectrophotometer (Shimadzu Model, UV-1601). One unit of invertase activity was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute under the assay conditions.

3. Results and discussion

During the electropolymerization of pyrrole, enzyme molecules were carried by pyrrole and supporting electrolyte. In order to understand which one was the predominant enzyme carrier, enzyme electrodes 2 and 3 were prepared. Their activities were determined at the same concentration of substrate (Table 1). In the case of EE3, since pyrrole polymerization was carried out before immobilization step; the only enzyme carrier was the supporting electrolyte. For EE2, both pyrrole and supporting electrolyte carry enzyme to the electrode surface. As seen from Table 1, the invertase activity of enzyme electrode 2 was 10 times higher than that of enzyme electrode 3. In addition to this, enzyme electrode 2 exhibited higher V_{max} compared to EE3. One can conclude that enzymes are mostly carried to electrode surface by the diffusion of pyrrole. Higher activity of invertase immobilized on the EE2 films also means more enzyme molecules entrapped compared to enzyme electrode 3.

Whether immobilized invertase is removed or not by the reduction of polymer synthesized in the presence of invertase was investigated. Firstly, enzyme electrode was prepared and invertase activity was calculated. After that, enzyme electrode was put into the electrolysis cell containing acetate buffer and SDS as the supporting electrolyte. It was reduced for 60 min. Activity of enzyme electrode was again determined. Immobilized invertase activity was not changed before and after the reduction process. No enzyme activity was found in the electrolysis solution. As a result, removal of immobilized invertase from the matrix is not possible by a simple electrochemical reduction process.

3.1. Morphologies of the films

Scanning electron microscopy (SEM) (JEOL JSM-6400) was used to examine the surface morphologies of the polymer films where enzyme was entrapped. The surface morphologies of these films were completely different compared to the films prepared in the absence of invertase. Cauliflower-like structure was noticeably damaged when invertase was entrapped in the matrix (Fig. 1). Moreover, invertase clusters were observed in the solution side of the films, especially for electrode 2 (Fig. 1a) and electrode 4

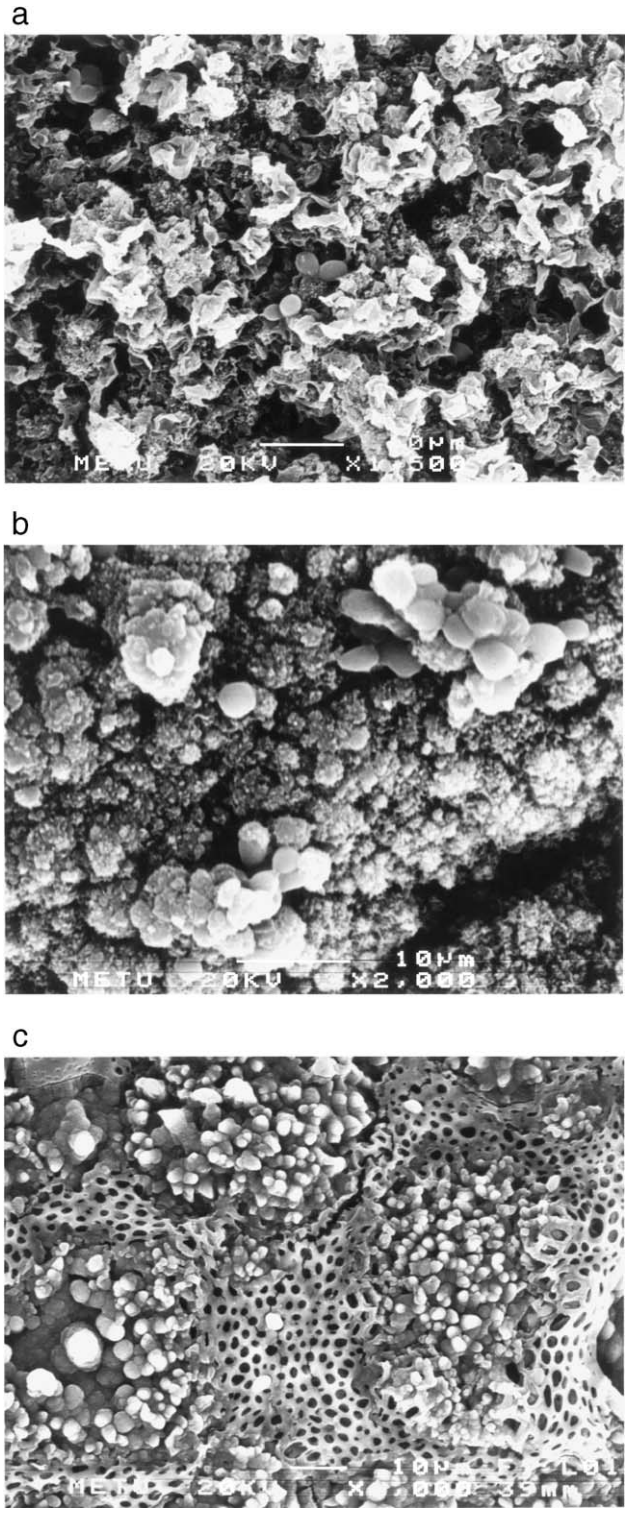


Fig. 1. Scanning electron micrographs of (a) solution side of EE2, (b) solution side of EE4, and (c) solution side of EE3.

Table 2
Kinetic parameters for hydrolytic breakdown of sucrose to glucose and fructose

Polymer matrix	K_m (mM)	V_{max} (μmol min^{-1} $electrode^{-1}$)
(EE1) PPy/invertase	28.2	4.17
(EE2) PMTM/PPy/SDS/invertase	30.2	3.15
(EE3) Pt(PMTM/PPy)/SDS/inv	26.2	1.16
(EE4) Pt(PMTM/PTh)/PPy/SDS/inv	22.5	3.17
(EE5) Pt(PTh)/PPy/SDS/inv	27.3	3.88

(Fig. 1b). On the other hand, the original structure of electrode 3 was not significantly damaged (Fig. 1c), as supported by lower kinetic parameters.

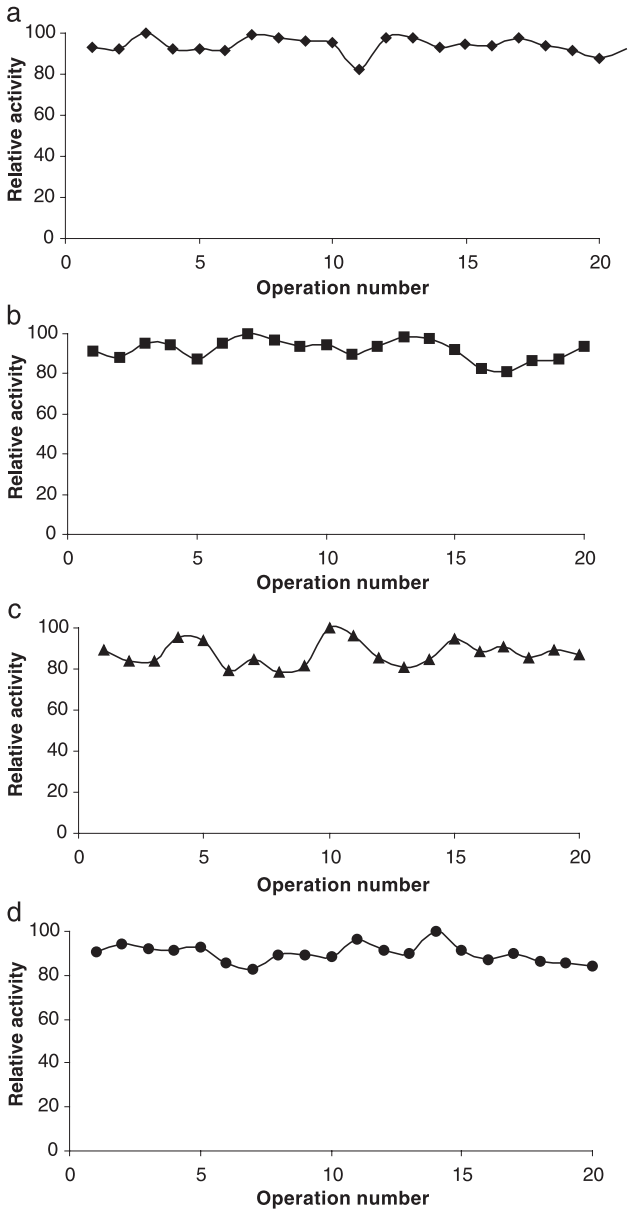


Fig. 2. Operational stability of (a) EE2, (b) EE3, (c) EE4, and (d) EE5.

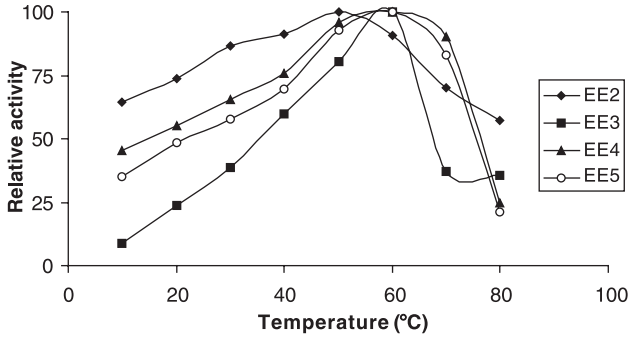


Fig. 3. Optimum temperature of enzyme electrodes.

3.2. Kinetic studies of immobilized invertase

Kinetic studies of the immobilized invertase were performed at various concentrations of sucrose. Maximum velocity, V_{max}, and the apparent Michaelis–Menten constant, K_m, [15] were found from the Lineweaver–Burk plot [16]. The calculated parameters are given in Table 2. Although different matrices were used, the K_m values were in the same order. The highest V_{max} was observed for electrode 1. The values obtained for electrodes 2, 4 and 5 are comparable with that of 1. In contrast, V_{max} value of electrode 2 was about 3.5 times lower than that of EE1.

3.3. Operational stability of the enzyme electrodes

One of the most important parameters to be considered in enzyme immobilization is operational stability. The stability of enzyme electrodes in terms of repetitive uses was studied. Applying more than 20 successive measurements revealed very small losses in the activity (Fig. 2). The enzyme activities were almost stable during the 20 experiments performed at 25 °C, on the day of immobilization.

3.4. Effect of temperature on the enzyme electrode response

The activity of the invertase is strongly dependent on temperature, with the optimum temperature being observed between 50 and 60 °C. As the temperature increases continuously after the optimum temperature, the structure of the enzyme becomes altered and its catalytic properties are reduced and eventually destroyed. The effect of temperature on enzyme electrodes was investigated and is given in Fig. 3. Maximum activity was found at 50 °C for enzyme electrode 3. However, optimum temperature of immobilized invertase was shifted to the 60 °C for enzyme electrodes 2, 4 and 5.

4. Conclusion

In this study, the immobilization of invertase in conducting copolymer matrices was achieved by electrochemical

polymerization procedures. The major enzyme carrier during immobilization was determined as the pyrrole monomer. It was also shown that enzymes can be entrapped in conducting polymers, here polythiophene, which cannot be synthesized in aqueous medium. The effect of reduction on the removal of immobilized invertase activity was also investigated. Kinetic parameters, operational stability, optimum temperature and morphology of enzyme electrodes were investigated.

Acknowledgements

This work is partially supported by BAP-2002-01-03-01 and TUBITAK Research Fund TBAG-2221 (102T116).

References

[1] A.A.A. Queiroz, M. Vitolo, R.C. Oliveira, O.Z. Higa, Invertase immobilization onto radiation-induced graft copolymerized polyethylene pellets, Radiat. Phys. Chem. 47 (6) (1996) 873–880.
[2] Y. Chen, E.T. Kang, K.G. Neoh, K.L. Tan, Covalent immobilization of invertase onto the surface-modified polyaniline from graft copolymerization with acrylic acid, Eur. Polym. J. 36 (2000) 2095–2103.
[3] P. Monsan, O. Combes, Application of immobilized invertase to continuous hydrolysis of concentrated sucrose solutions, Biotechnol. Bioeng. 26 (1984) 347–351.
[4] U. Akbulut, S. Sungur, S. Pekyardimci, Polyester film strips coated with photographic gelatin containing immobilized invertase, Macromol. Rep. A 28 (1991) 239–247.
[5] M. Marek, O. Valentova, J. Kas, Invertase immobilization via its carbohydrate moieties, Biotechnol. Bioeng. 26 (1984) 1223–1226.
[6] J. Mansfeld, M. Förster, A. Schellenberger, H. Dautzenberg, Immobilization of invertase by encapsulation in polyelectrolyte complexes, Enzyme Microb. Technol. 13 (1991) 240–244.
[7] P.A. Dickensheets, L.F. Chen, G.T. Tsao, Characteristic of yeast invertase immobilized on porous cellulose beads, Biotechnol. Bioeng. 19 (1977) 365–375.
[8] C. Simioneseu, M. Popa, S. Dumitru, Immobilization of invertase on the diazonium salt of 4-aminobenzoylcellulose, Biotechnol. Bioeng. 28 (1987) 198–203.
[9] K. Imai, T. Shiami, K. Uchida, M. Miya, Immobilization of enzyme onto poly (ethylene-vinyl alcohol) membrane, Biotechnol. Bioeng. 28 (1986) 198–203.
[10] F. Selampinar, U. Akbulut, M.Y. Özden, L. Toppare, Immobilization of invertase in conducting polymer matrices, Biomaterials 18 (1997) 1163–1168.
[11] N. Kizilyar, U. Akbulut, M.Y. Özden, L. Toppare, Y. Yağcı, Immobilization of invertase in conducting polypyrrole/polytetrahydrofuran graft polymer matrices, Synth. Met. 104 (1999) 45–50.
[12] S. Alkan, L. Toppare, Y. Yağcı, Y. Hepuzer, Immobilization of invertase in conducting thiophene-capped poly (methylmethacrylate)/polypyrrole matrices, J. Biomater. Sci., Polym. Ed. 10 (12) (1999) 1223–1235.
[13] A. Çırpan, S. Alkan, L. Toppare, Y. Yağcı, Y. Hepuzer, Conducting graft copolymers of poly (3-methylthienyl methacrylate) with pyrrole and thiophene, J. Polym. Sci., Polym. Chem. 40 (2002) 4131.
[14] N. Nelson, A photometric adaptation of the Somogyi method for the determination of glucose, J. Biol. Chem. 153 (1944) 375–380.
[15] L. Michaelis, M.L. Menten, Die Kinetik der Invertinwirkung, Biochem. Z. 49 (1913) 333–369.
[16] H. Lineweaver, D. Burk, The determination of enzyme dissociation constant, J. Am. Chem. Soc. 56 (1934) 658–666.