Application of UV-Curable Diazoresin. II. Characteristics of the Immobilized Glucose Oxidase Membrane*

JUI-HSIANG LIU,† MING-YI CHEN, and YUH-CHWEN CHANG

Department of Chemical Engineering, National Cheng Kung University, Tainan, Taiwan 70101, Republic of China

SYNOPSIS

The characteristics of the enzyme electrode, developed using the immobilized glucose oxidase membranes, were investigated. The enzyme membranes were prepared from poly(vinyl alcohol)-diazoresin and poly(vinyl alcohol)-sensitizer systems, cross-linked by means of ultraviolet light irradiation. Temperature and pH dependencies of the relative activity, stability in repeating usage, storage stability, and calibration plots were evaluated. The unstable phenomenon, found in the initial usage of the immobilized glucose oxidase membrane, which was prepared from poly(vinyl alcohol)-sensitizer system, was also investigated.

INTRODUCTION

Photosensitive polymers have been particularly interesting because they are being used as photoresists to make large-scale integrated circuits, printing plates, photocurable coatings,[1] photorecorders,[2] photoconductors,[3] energy exchange materials,[4] enzyme fixing materials,[5] photoetching groups in organic syntheses,[6] and photosensitizers[7] of organic syntheses.[8]

In our previous article,[9] we reported the preliminary results of the entrapment of glucose oxidase within poly(vinyl alcohol) membrane, crosslinked by means of ultraviolet (UV) light irradiation, in the presence of UV-sensitive diazoresin and benzoic acid and/or sodium benzoate sensitizers. Conditions of the immobilization, activity yields of the enzyme membrane, and effects of irradiation time on the degree of insolubility of the membranes were discussed.

The immobilization method is useful because of its easy handling and mild condition for immobilization of enzyme.

In this article, as the continuation of our previous study,[9] we present the characteristics of the enzyme electrode developed by the immobilized glucose oxidase membranes. Temperature and pH dependencies of the relative activity, storage stability, stability in repeating usage, and calibration plots were evaluated. The unstable phenomenon, found in the initial usage of the immobilized glucose oxidase membrane, which was prepared from poly(vinyl alcohol) containing a certain amount of benzoic acid and/or sodium benzoate, was also investigated.

EXPERIMENTAL

Materials

Commercially available PVA of $\bar{P}$ = 72,000 ($\bar{n}$ = 637, degree of hydrolyzation = 98%) was used without further purification. Glucose oxidase from Sigma Co. (3500 U/mg) and from Merck Co. (90 U/mg), glucose from Merck Co. (biochemical grade), benzoic acid from Merck Co. (GR grade), and sodium benzoate from Hayashi Co. (GR grade) were used without purification. Diazoresin was prepared according to the literature.[10]

Measurements

The IR spectra were recorded on a Jasco IR-810 grating IR sectrophotometer. The NMR spectra were recorded on a Brucker-100, high resolution NMR spectrometer. The UV spectra were recorded on Hitachi UV-200-20 spectrophotometer. Concen-

* For Part I, See Reference 9.

† To whom correspondence should be addressed.

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trations of oxygen dissolved in water were estimated by using a Suntex SD-70 D.O. (dissolved oxygen) meter.

Gelation of Poly(Vinyl Alcohol)

The PVA was gelled as follows. Membranes of about 60 μm in thickness were obtained by casting 10 wt % PVA solution, containing diazoresin or benzoic acid and/or sodium benzoate sensitizers, on a polytetrafluoroethylene plate dried at room temperature. Various amounts of sensitizer were used to evaluate the effects of sensitizer concentration on gel content and the optimum condition was estimated.[9] The membrane was irradiated with 8 W Camag UV lamp (main wavelength, 254 nm and 365 nm) at a distance of 3 cm at room temperature. Effects of irradiation time on degree of insolubility of the membrane for PVA–diazoresin (irradiated with λ_m = 365 nm lamp) and PVA–sensitizer (irradiated with λ_m = 254 nm lamp) systems were investigated.

Immobilization of Glucose Oxidase

PVA (0.6 g) in 8 mL phosphate buffer solution (0.03 M, pH = 7.05) was heated to 60°C with stirring. After PVA was dissolved completely, the PVA buffer solution was cooled at room temperature. To the PVA buffer solution was added 2 mL 0.03 M (pH = 7.05) phosphate buffer solution containing a given amount of glucose oxidase. Membranes were prepared by casting the PVA buffer solution on a polytetrafluoroethylene plate and then were dried at room temperature. The enzyme-entrapping membrane was cut into 1.7 × 1.7 cm squares and then was irradiated with an 8 W Camag UV lamp (main wavelength of 254 nm and 365 nm were used) at a distance of 3 cm at room temperature. The enzyme-entrapping membranes thus obtained were stored in a refrigerator to be subjected to the series.

Estimation of Enzyme Activity

Enzyme activity was estimated by measuring the change (ppm) or change rate (ppm/min) of dissolved oxygen concentration in buffer solution while the substrate (glucose) was added. Enzyme membrane was fixed at the surface of cathode with an O-ring; the schematic diagram for enzyme analysis was shown in our previous article.[9]

Glucose oxidase 20 mg was dissolved in 10 mL phosphate solution (0.03 M, pH = 7.05) to prepare an enzyme solution. Enzyme solution (0.25 mL) thus obtained was added to 50 mL phosphate buffer solution, the D.O. value will appear stable within a certain time. Glucose solution (0.5 mL, 10%) was then added and the initial change rate of the D.O. value (ppm/min) in the system was recorded.

The activity of the immobilized enzyme membranes was calculated at 25°C in 50 mL pH = 7.05 phosphate buffer solution. Glucose solution (2 mL) in various concentrations were added and the activity of the enzyme membranes was then estimated by measuring the change (ppm) of D.O. value in the buffer solution.

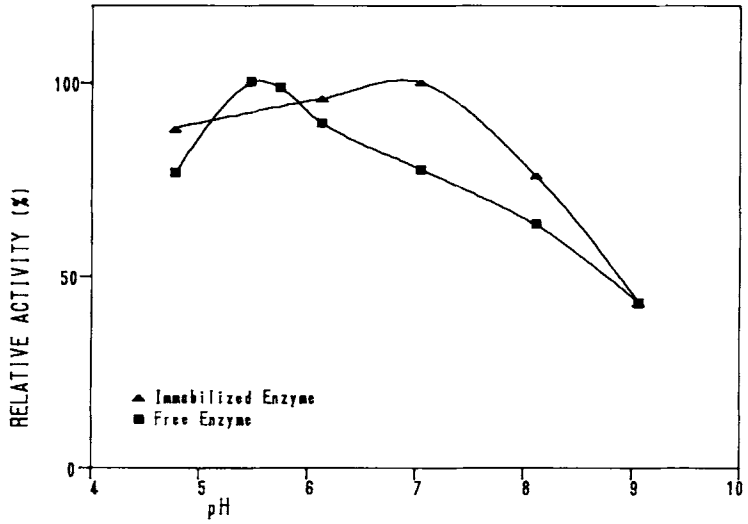

Figure 1 The pH-activity curve for the PVA–diazoresin system. Reaction temperature = 25°C; glucose = 10%.

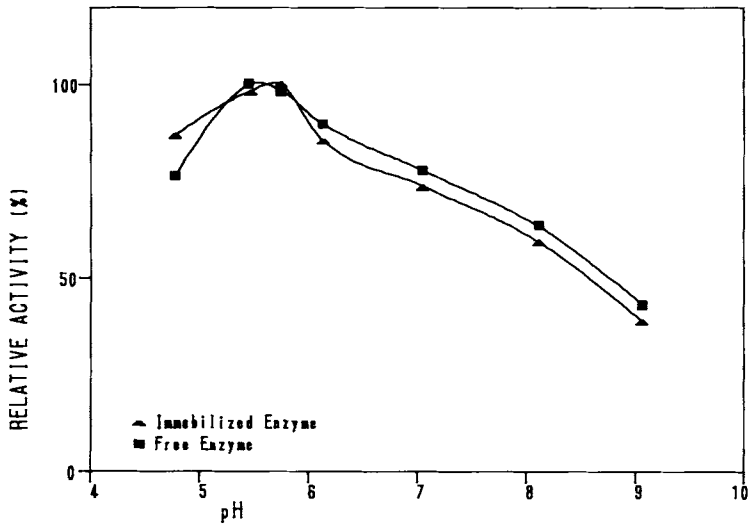

Figure 2 The pH-activity curve for the PVA–sensitizer system. Reaction temperature = 25°C; glucose = 10%.

RESULTS AND DISCUSSION

pH Dependence

Various characteristics of glucose oxidase immobilized under the conditions described in the previous section were compared to those of the native one using glucose as a substrate. The pH dependence of the relative activity of the glucose oxidase in PVA–diazoresin and PVA–sensitizer membranes are compared with those of the native enzyme, and the results are shown in Figures 1 and 2. The reactions were carried out in a phosphate buffer solution of pH 4.8–9.0. As shown in Figure 1, the optimum pH value for the enzyme membrane immobilized by PVA–diazoresin is around 7.0, which shifted from the acid side to a mild value as compared with that of the native enzyme. As can be seen in Figure 2, this tendency was also observed for the PVA–sensitizer system.

During the enzyme reaction, gluconic acid was produced and this will cause the decreasing of the pH value within the membranes. The optimum pH value detected in the outer bulk solution hence shifted to higher pH value in both systems. Because of the difference of the hydrophilicity between the

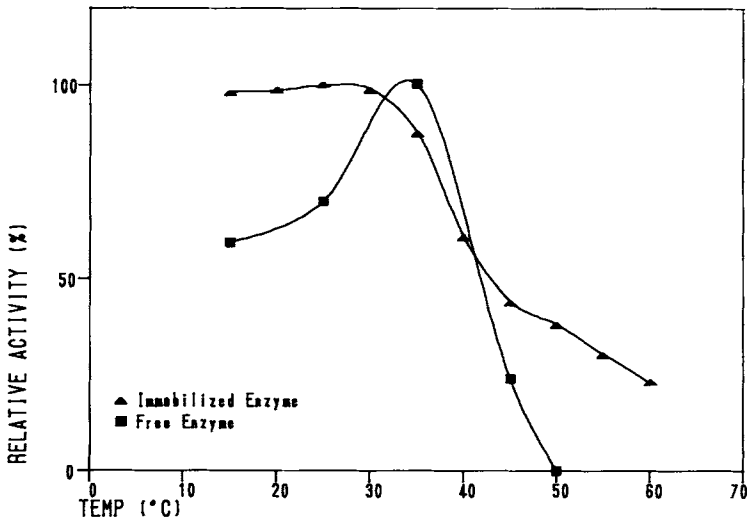

Figure 3 Temperature–activity curve for the PVA–diazoresin system. pH = 7.05; glucose = 10%.

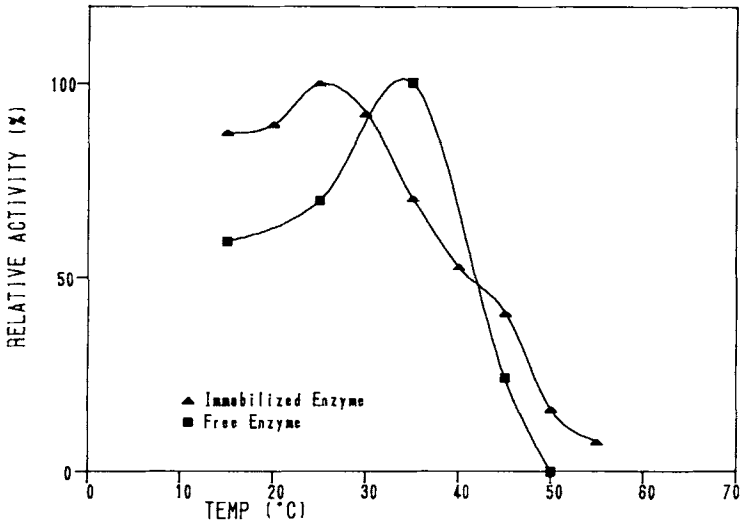

Figure 4 Temperature–activity curve for the PVA–sensitizer system. pH = 7.05; glucose = 10%.

systems, the effect of pH dependence on the PVA–diazoresin system is larger than that of the PVA–sensitizer system.

Temperature Effect

Figures 3 and 4 show the temperature dependencies of the relative activity for PVA–diazoresin and PVA–sensitizer systems, respectively. The thermal stability was examined in buffer solution (pH 7.05) at a given temperature. The optimum temperature in each system is found to be shifted to a lower temperature (20–30°C) as compared with that of the native enzyme.

On both sides of the optimum temperature, the immobilized enzyme maintains higher relative activity than the native enzyme does. As shown in the figures, the native enzyme lost its activity completely at about 50°C. The enzyme immobilized in PVA–diazoresin had higher thermal stability. Operating the systems at the optimum mild temperature will also increase the life of the electrode.

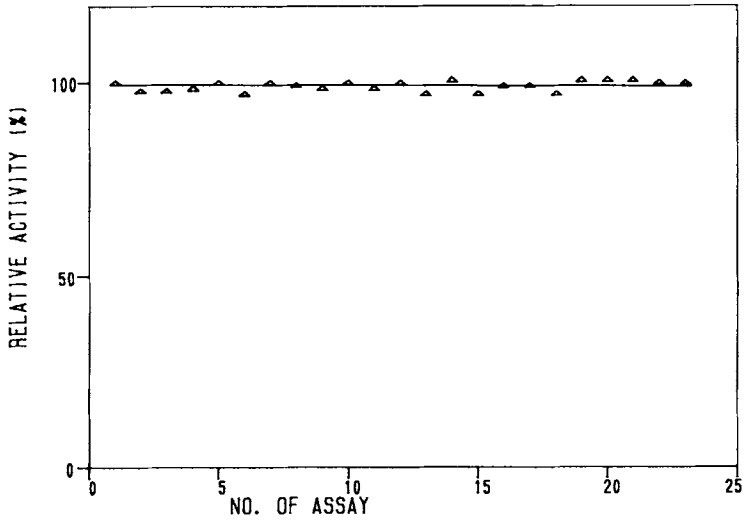

Figure 5 Activity in repeating usage for the PVA–diazoresin system. Reaction temperature = 25°C; pH = 7.05; glucose = 1%.

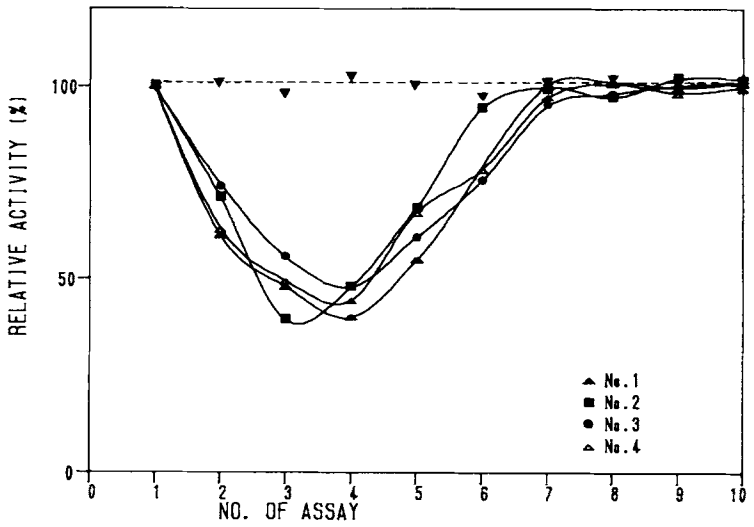

Figure 6 Activity in repeating usage for the PVA–sensitizer system. Reaction temp. = 25°C; pH = 7.05, glucose = 10%; (dashed line) with preswelling; (solid line) without preswelling.

Stability in Repeating Usage

The stability in repeating usage was examined as follows: The immobilized enzyme was fixed at the surface of cathode with the O-ring as described in our previous article.[9] Relative activity was detected at 25°C in buffer solution (pH = 7.05) using 1% glucose as a reaction substrate.

Figure 5 shows the results on the stability of the PVA–diazoresin system for the repeating usage. As can be seen, the relative activity remained unchanged during the repeating usages. As shown in Figure 6, an unstable phenomenon was found in the initial usage of the immobilized glucose oxidase membrane prepared from the PVA–sensitizer system. The unstable phenomenon disappeared, however, when the enzyme electrode was kept in buffer solution (pH = 7.05) at 25°C for 2 h before the first time usage (dashed line in Fig. 6).

We suggest that the optimum enzyme conformation deformed and that the part of the linear PVA in membrane dissolved as the crosslinked PVA

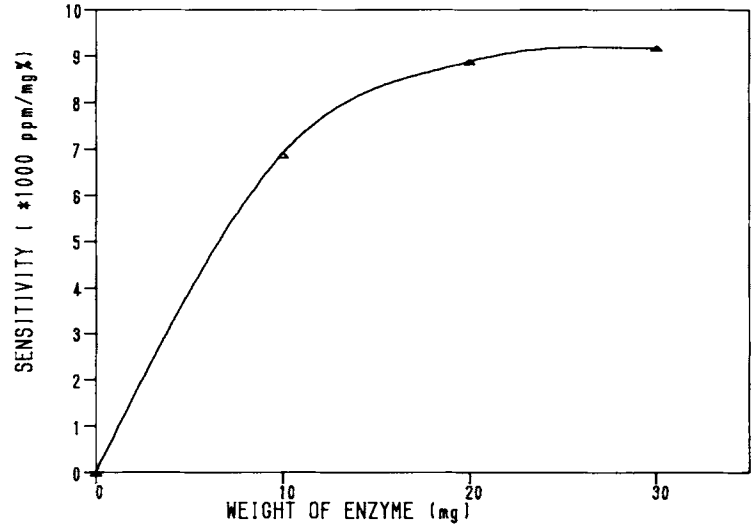

Figure 7 Dependence of sensitivity of the PVA–diazoresin membrane on concentration of enzyme. Reaction temperature 25°C; pH = 7.05.

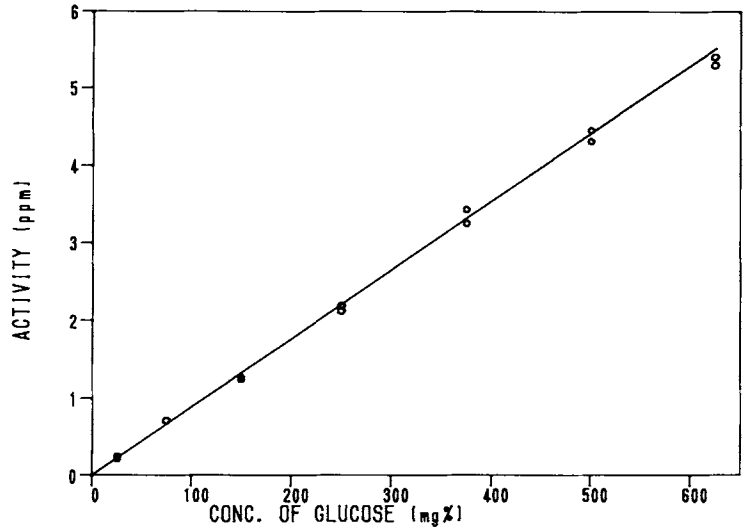

Figure 8 Calibration plot of activity vs. glucose concentration for the PVA–diazoresin system. Enzyme = 23.5 mg/g gel; reaction temp. = 25°C; pH = 7.05.

membrane swelled in the buffer solution during the usage of the enzyme electrode. In general, the optimum conformation of the enzyme will be fixed during the drying process of the membranes and the swelling of the crosslinked membrane will cause the change of the enzyme conformation. The change of the enzyme conformation and/or the steric hinderance from the soluable part of the uncrosslinked linear PVA may decrease the stability of the enzyme activity. Preswelling and sufficient rinse of the membrane before the first time usage will remove the steric hinderance and will prevent the deforming of the enzyme conformation, and the unstable phenomenon will thus disappear.

Calibration Plot and Storage Stability

The dependence of the sensitivity of the enzyme membrane prepared from PVA–diazoresin on the concentration of glucose oxidase was examined at 25°C in pH 7.05 buffer solution. The result is summarized in Figure 7. As can be seen, the sensitivity

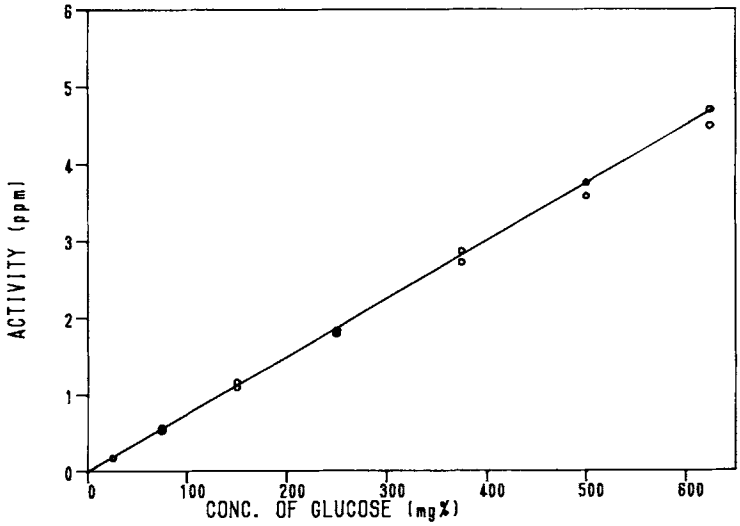

Figure 9 Calibration plot of activity vs. glucose concentration for the PVA–sensitizer system. Enzyme = 23.5 mg/g gel; reaction temperature = 25°C; pH = 7.05.

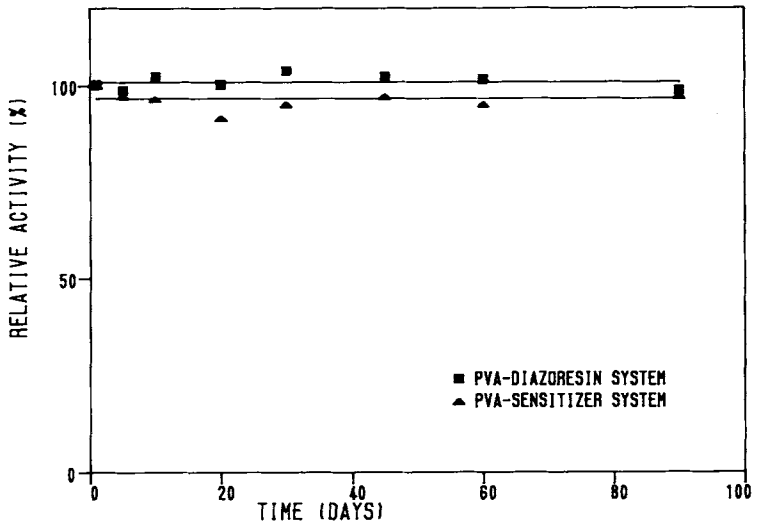

Figure 10 Storage stability of the dry membranes. Both dry membranes were stored in a freezer.

of the enzyme membrane increased with increasing the weight of the enzyme used, levelling off at about 30 mg.

Calibration curves on glucose standard solutions obtained with the enzyme membranes of PVA–diazoresin and PVA–sensitizer systems are shown in Figures 8 and 9, respectively. The linearity range (about 25–625 mg/dL) can be observed in those two systems. In these ranges the correlation value is satisfactory, larger than 0.99, as is the precision, given as "pooled standard deviation."

The storage stability of the enzyme membranes were examined by storing them in a freezer (dry state) and/or in buffer solution (wet state) at room temperature. The results are shown in Figures 10 and 11. In Figure 10, both the PVA–diazoresin and the PVA–sensitizer systems were kept dry in the freezer, and the relative activity remained unchanged within 90 days. The wet storage stability was examined in 0.03 M phosphate buffer solution (pH = 7.05) and the wet enzyme membrane was stored in a refrigerator at 3°C. As shown in Figure

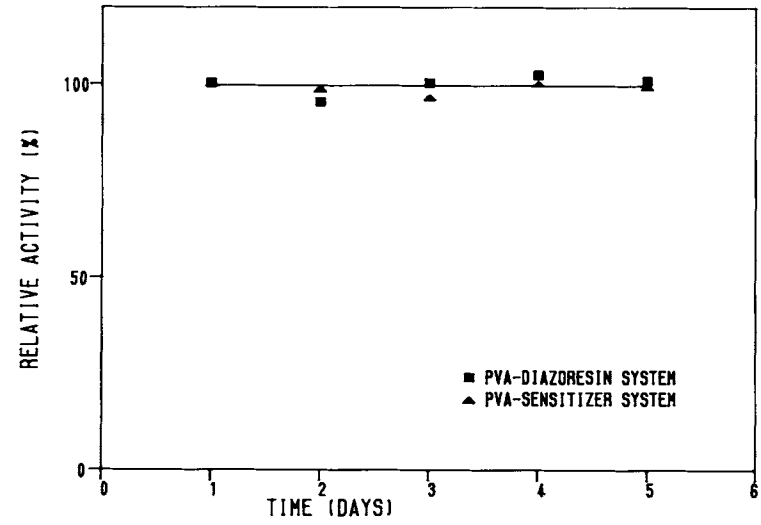

Figure 11 Storage stability of the wet membranes. Both wet membranes were stored in pH 7.05 buffer solution at 3°C.

11, the relative activity remained unchanged within 5 days.

CONCLUSION

The glucose oxidase was immobilized into poly(vinyl alcohol) membrane in the presence of UV light sensitive diazoresin and benzoic acid and/or sodium benzoate sensitizers. Activity yields of 39.98 and 35.30 for PVA–diazoresin and PVA–sensitizer systems were obtained, respectively. An effective enzyme electrode, using the immobilized glucose oxidase membrane, was developed. The steric structures of the two UV curable PVA after UV irradiation are quite different, and the response time of 2–5 min and 1–3 min for PVA–diazoresin and PVA–sensitizer were obtained. The optimum pH value for both enzyme membranes are found at around 7.0, which shifted from the acid side to the mild value as compared with that of the native enzyme. Because of the difference of the hydrofilicity between the systems, the effect of pH dependence on the PVA–diazoresin system is larger than that of the PVA–sensitizer system.

The optimum temperature in both systems was found to shift to lower temperatures (20–30°C). On both sides of the optimum temperature, the immobilized enzyme maintained higher relative activity than the native enzyme does. Operation of the systems at the optimum mild temperature and pH value (pH around 7.0) will also increase the life of the electrode. The relative activity remained unchanged during repeated usage. The unstable phenomenon, found in the initial use of the immobilized glucose oxidase membrane prepared from the PVA–sensitizer system, disappeared, while the enzyme electrode remained stable in the buffer solution (pH = 7.05) at 25°C for 2 h. The sensitivity of the enzyme membrane increased with increasing the weight of the enzyme used, levelling off at about 30 mg. The linearity range (about 25–625 mg/dL) can be observed in both systems. The storage stability of the enzyme membranes were also examined. The relative activity of the dry membranes remained unchanged within 90 days, and the wet storage stability remained unchanged within 5 days.

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