Studies on the Physical Properties of Polyethylene-*g*-Acrylic Acid to Immobilizing Glucose Oxidase

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Synopsis

A new type of polymer support for immobilizing glucose oxidase was investigated. The starting copolymer was acrylic-acid-grafted polyethylene (PE-*g*-AA). The copolymer was prepared with gamma ray irradiation induced grafting under controlled conditions utilizing post-irradiation procedures. A number of factors were examined. They were: (1) the effect of the hydrophilic/hydrophobic nature of the support on the enzyme membrane activity; (2) the Michaelis constant (k_m); (3) the thermal stability, the rate constant for the thermal denaturation, and the activation energies for the denaturation of immobilized and soluble enzyme; and (4) the physical properties of the support affecting the immobilized enzyme activity. An attempt was made to immobilize the enzyme-utilizing carbodiimide (CMC) as the coupling agent. This immobilization method resolves in a high enzyme activity. The hydrophilicity of the support and the activity of the enzyme membrane were proportional to the degree of grafting. When the temperature was higher than 60°C, the immobilized glucose oxidase was less sensitive to thermal inactivation than the native enzyme. Various polyethylene was studied; the LLDPE was preferred. The type of polyethylene, the thickness, kind of grafting monomers, and the degree of grafting would influence the activity of the enzyme membrane.

INTRODUCTION

A large number of native and synthetic polymers have been used as solid supports for the attachment of enzymes. The selection of the supports depends on the method of immobilization. Owing to the importance of the existence of suitable reactive groups, hydrophilic polymers were selected as enzyme immobilized supports.

Hydrogels normally show a high affinity for proteins which can be entrapped in the pores of the gel. This renders difficulty for the diffusion of the active sites and their reactions with substractes. With hydrophobic supports, coupling generally takes place on the surface. The effect of the hydrophobic nature of such support system on the enzyme activity and the relatively small number of reactive groups on the surface tend to make these systems less acceptable. However, the grafted substance should also be hydrophilic in nature, in order to counteract hydrophobic interactions between the hydrophobic carrier PE and the protein, which could lead to a decrease in biological activity. Hence, graft copolymer was selected to be the enzyme-immobilized supports.

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In this study a polymeric support, which was obtained by grafting acrylic acid (AA) or methacrylic acid (MAA) onto polyethylene (PE) film with the aid of Co 60 gamma ray induced graft polymerization. The advantage of this kind of support was to provide the functional group onto the hydrophobic polyethylene. That the water-soluble 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMC) acted as a condensing agent to immobilize glucose oxidase onto PE-*g*-AA was a successful method.[1] The use of graft copolymers as enzyme supports has been reported.[2-8] However, they did not study the effect of physical properties of the graft copolymers. Therefore, we will investigate various physical properties of the graft copolymers which affect the enzyme membrane activity.

EXPERIMENTAL

Materials

β-D-glucose oxidase (E.C. 1.1.3.4. from *Aspergillus Niger,* type X), was a product of Sigma Chemical Co. All the other chemicals used in this study were of reagent grade.

The materials used as grafting substances included: (1) low density polyethylene film (LDPE) with thickness 0.02 mm; (2) linear low density polyethylene (LLDPE) with thickness 0.02 and 0.035 mm; and (3) ethylene vinylacetate-co-polyethelene (17% EVA) with thickness 0.02 mm. These films were kindly provided as a gift from USI Fast Corp. The CMC was purchased from Merck.

Apparatus

The sensor system consisted of a dissolved oxygen electrode (YSI Model 5750, BOD bottle probe), D.O. Meter (Suntex Model SD-60) and a recorder (Eyela Model TR-250). The scanning electron microscope (SEM) (HITAHI-570) and transmission electron microscope (TEM) (JEOL JEM 200 (x)) were used to analyze the morphology of the graft copolymer. The electric resistance of the grafted film was measured in 40% KOH solution at 25°C using an ohmmeter (LCR Meter ZM-341 NF Electronic Instruments) working at 1000 Hz.

EXPERIMENTAL PROCEDURES

Preparation of Graft Copolymer

The preparation of PE-*g*-AA and PE-*g*-MAA membrane was carried out according to the procedure of Hsiue and Huang.[9] A cobalt 60 gamma source at National Tsing Hua University, Hsinchu, Taiwan, R. O. C. was used. The degree of grafting was obtained from the weight increase of the films according to the following equation[9]:

$$\% \text{ graft} = (W_1 - W_0)/W_0 \times 100$$

where W_0 is the initial weight of parent film and W_1 is the weight of grafted film.

Preparation of Immobilized Glucose Oxidase Membrane

The procedure was the same as that reported by Hsiue and Wang.[1] Various types of graft copolymers ($\frac{5}{8}$ in. in diameter) were used to couple glucose oxidase in pH 3.5 acetate buffer solution using 2 mg glucose oxidase and 30 mg CMC.

Preparation of Biosensors

The biosensors were prepared by combining the enzyme immobilized membranes with the electrode.[1]

Measurement of Graft Copolymer Physical Properties

Water Contents

The water contents were obtained from the weight increase of the films according to the following equation:

$$\text{water contents } \% = (W_1 - W_0)/W_0 \times 100$$

where W_0 is the weight of the dried film and W_1 is the weight of water-saturated grafted film.

Electric Resistance

The electric resistance of the grafted film was measured in 40% KOH solution at 25°C using an ohmmeter. The sample was soaked for 24 h in the potassium hydroxide solution prior to being interposed between two platinized platinum electrodes.

Michaelis Constant (k_m)

The activity of natural glucose oxidase and enzyme immobilized membrane was examined in glucose solution of various concentrations.

Thermal Denaturation

Temperature in the range from 50 to 70°C was maintained at a constant value to within 0.05°C and the free glucose oxidase or immobilized glucose oxidase was exposed for a period up to 80 min. The activity was measured.

RESULTS AND DISCUSSION

The Effect of Degree of Grafting on Enzyme Membrane

The Relationship between Degree of Grafting and Electric Resistance

The electric resistance of PE-g-MAA was higher than that of PE-g-AA at the same degree of grafting. LLDPE-g-AA at approximately 30% degree of

grafting was penetrated, but LLDPE-*g*-MAA was penetrated at nearly 60% (Fig. 1). The relationship between electric resistance and degree of grafting of LDPE system is shown in Figure 2. LDPE-*g*-AA and LDPE-*g*-MAA penetrated at 40 and 70% degrees of grafting, respectively. When the degree of grafting was too high, both grafted polyethylenes would crack. The suitable grafted amounts of monomer were acrylic acid 80% and methacrylic acid 120%.

The Relationship between Degree of Grafting and Enzyme Membrane Activity

The relationship between LLDPE, LDPE grafted with acrylic acid or methacrylic acid, and their activity is shown in Figures 3 and 4, respectively. As the degree of grafting increased, both activities increased rapidly. It was obvious that the activity of grafted acrylic acid type was much higher than that of the methacrylic acid type. Comparing the profile of activity with electric resistance, as the film was penetrated the activities increased. The enzyme activity of PE-*g*-MAA being less than that of PE-*g*-AA was caused by the stereo resistance of the methyl group. Different types of polyethylenes were grafted with acrylic acid. The enzyme activity of these enzyme membrane is shown in Figure 5. For the same degree of grafting, the LLDPE film had the best results. The thin membrane had better enzyme activity than that of the thick membrane. Among all PE of equal thickness, LLDPE-*g*-AA was the best. Now, we can conclude that LLDPE (0.02 mm) is the best type of PE for immobilizing glucose oxidase.

The Relationship between Water Content and Enzyme Activity

As the degree of grafting increased, the water content increased (Fig. 6). The relationship between water content and enzyme activity is shown in Figure

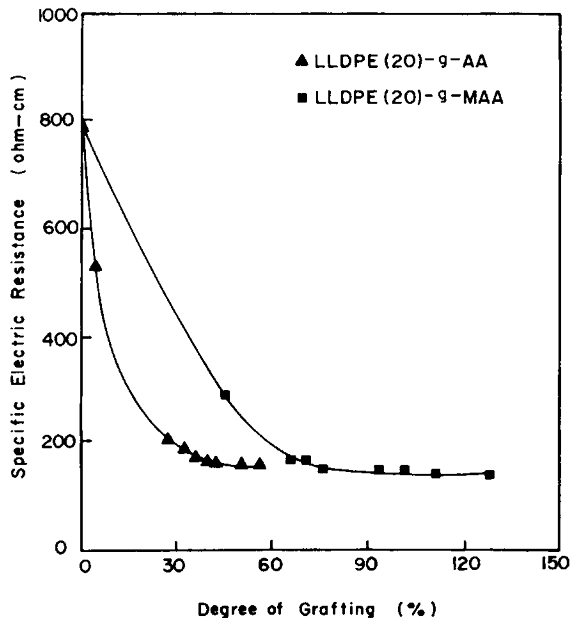

Fig. 1. Relationship between specific electric resistance and the degree of grafting of LLDPE with acrylic acid and methacrylic acid.

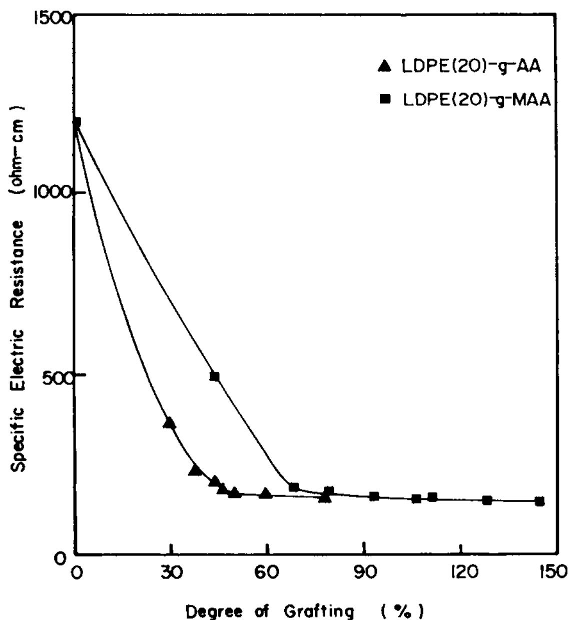

Fig. 2. Relationship between specific electric resistance and the degree of grafting of LDPE with acrylic acid and methacrylic acid.

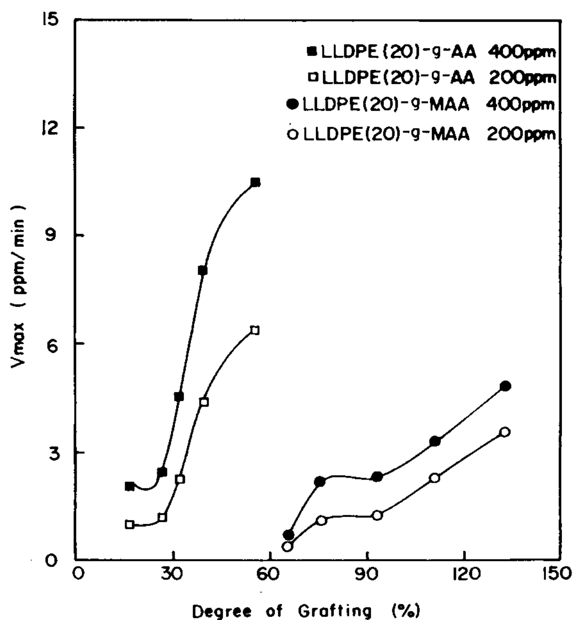

Fig. 3. Activity as a function of the degree of grafting of LLDPE with acrylic acid and methacrylic acid.

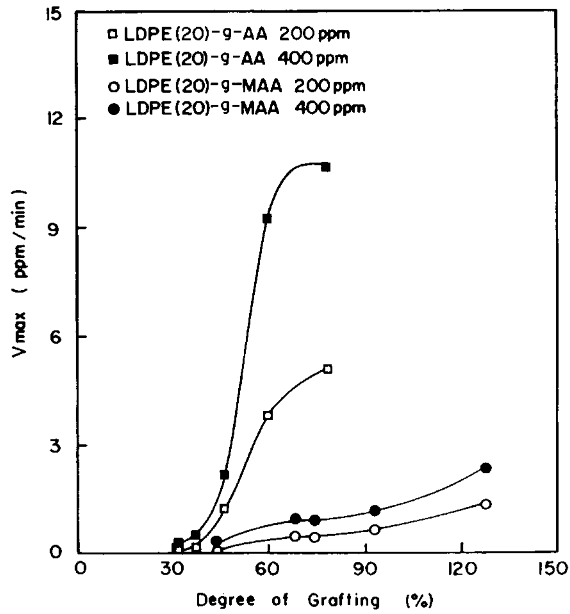

Fig. 4. Activity as a function of the degree of grafting of LDPE with acrylic acid and methacrylic acid.

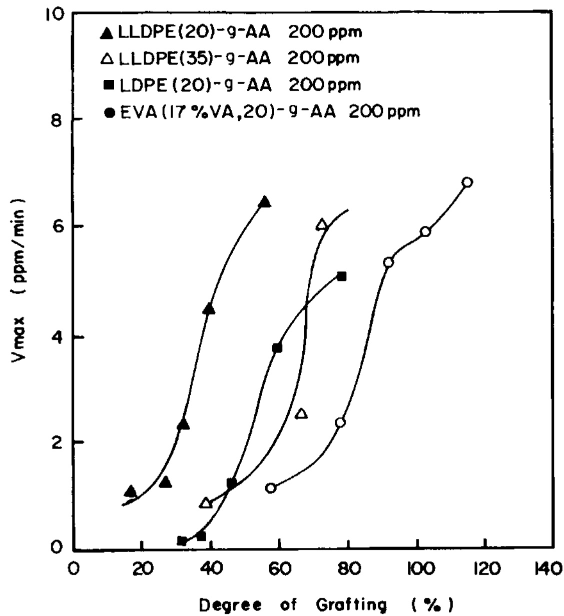

Fig. 5. Activity as a function of the degree of grafting of various PE grafted with acrylic acid.

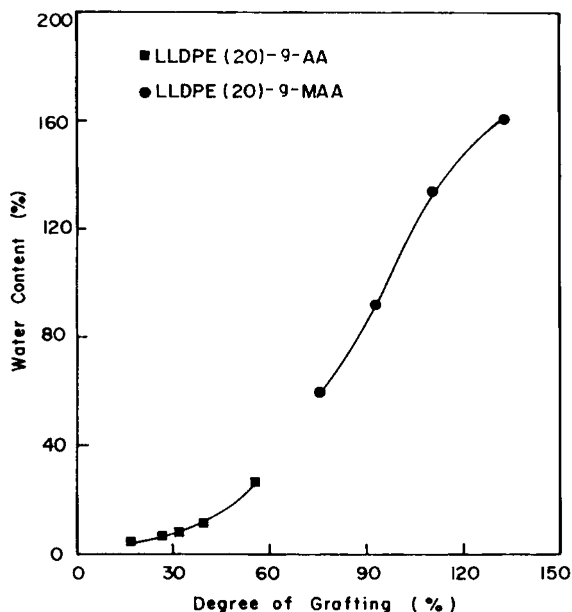

Fig. 6. Relationship between water content and the degree of grafting of LLDPE with acrylic acid and methacrylic acid.

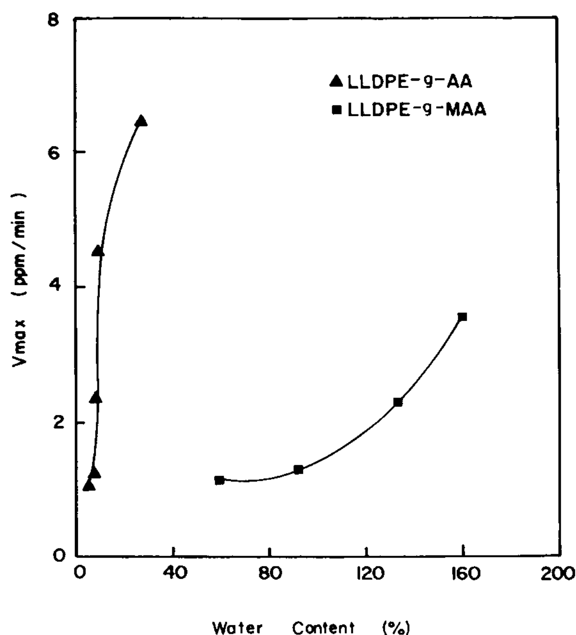

Fig. 7. Relationship between activity and water content. Each support was treated with 20 mg CMC and 3 mg GOD at 4°C for 16 h. The immobilization pH value was 3.5.

7. The profile is similar to that in Figure 1. The high degree of grafting did not decrease the enzyme activity. This means that carboxylic acid group acidity would not affect the enzyme activity. Increasing the degree of grafting would increase the number of the carboxylic acid groups, which in turn would make the film more hydrophilic, longer in chain length, and more flexible. All these factors increased the enzyme activity. Therefore, the thickness of PE film, the types of monomers, and the degree of grafting determined the immobilized enzyme membrane activity.

Graft Copolymer Morphology Study

Figure 8 shows the SEM photographs of LLDPE-*g*-AA (72.5%) membranes with 0.035 mm thickness on the surface and cross section. Comparison between

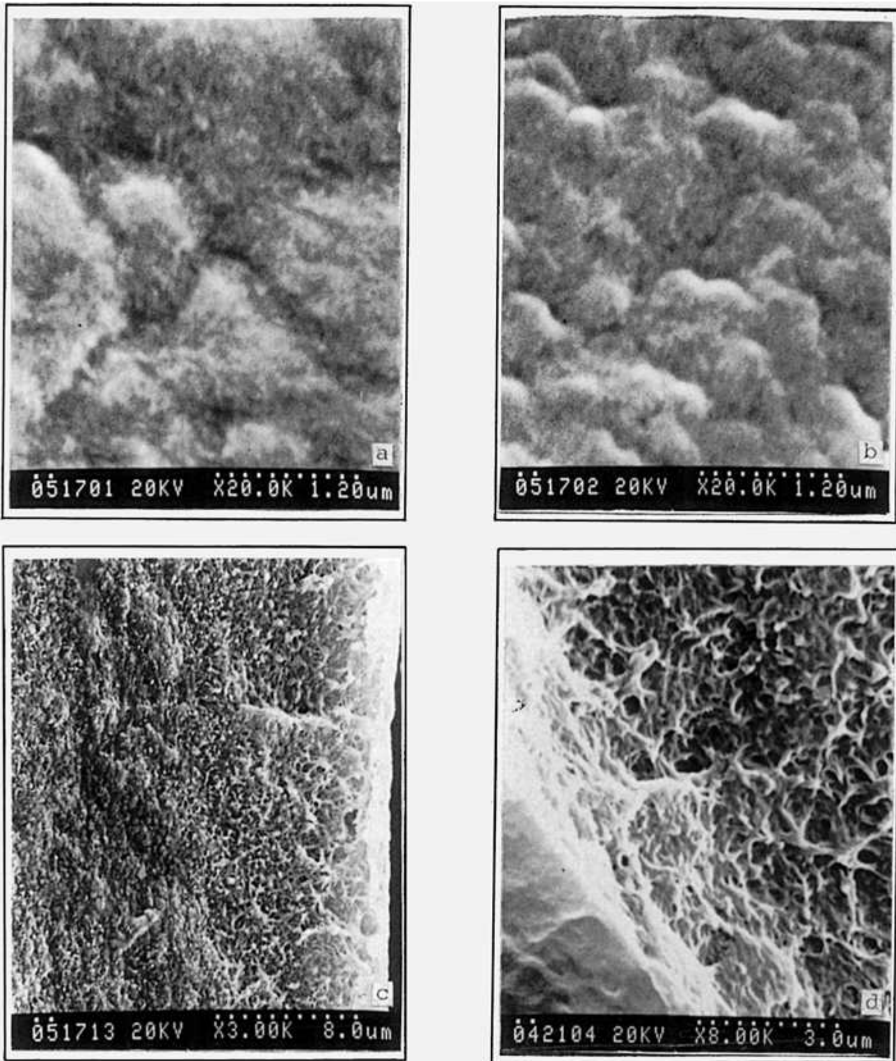

Fig. 8. SEM photographs of LLDPE-*g*-AA membranes on the surface (a, b) and on the cross section (c, d): (a) LLDPE (× 20,000); (b) LLDPE-*g*-AA (× 20,000); (c) LLDPE-*g*-AA (× 3000); (d) LLDPE-*g*-AA (× 8000).

(a) and (b) showed that grafted membrane was a dense film. Grafted films were more crude than the polyethylene. Photographs (c) and (d) showed the cross section of LLDPE-*g*-AA. It had more grafted polyacrylic acid on the surface. In general, the graft copolymer was a normal distributed grafted product.

Figure 9 shows the TEM photographs of LLDPE-*g*-AA (72.5%) with a thickness of 0.035 mm. The black parts represent carboxylic acid groups, and the white parts represent the polyethylene. Hence, LLDPE-*g*-AA was homogenerous by distributed grafted film.

Searching of Enzyme Membrane Kinetics

Michaelis Constant (k_m) *of the Immobilized Enzyme Membranes*

Lineweaver–Burk plots of the native glucose oxidase and immobilized glucose oxidase are shown in Figure 10. A good linear relationship was obtained. This result suggests that the decomposition of glucose through the glucose oxidase immobilizing graft copolymer membrane can be interpreted as a normal enzymatic reaction. The kinetic data obtained from the intercepts of axis in Figure 10 were: k'_m (immobilized) = 0.0017 *M;* V_{max} = 294 ppm/min; k_m (native) = 0.0025 *M;* and V_{max} = 887 ppm/min. The reciprocal of Michaelis constant $1/k_m$, which was evaluated as the affinity constant between the substrate and enzyme molecules, was slightly larger in the system of enzyme immobilizing membrane. PE-*g*-AA that constructed the graft copolymer membrane was a barrier to the approach of substrate molecule acting on the immobilized enzyme molecule. Consequently, the affinity constant became higher in the enzyme immobilizing membrane.[10]

Thermal Denaturation of the Immobilized Enzyme Membrane

Typical plots of the normalized enzyme activity vs. time are shown in Figures 11 and 12 for native and immobilized glucose oxidase, respectively. The first-

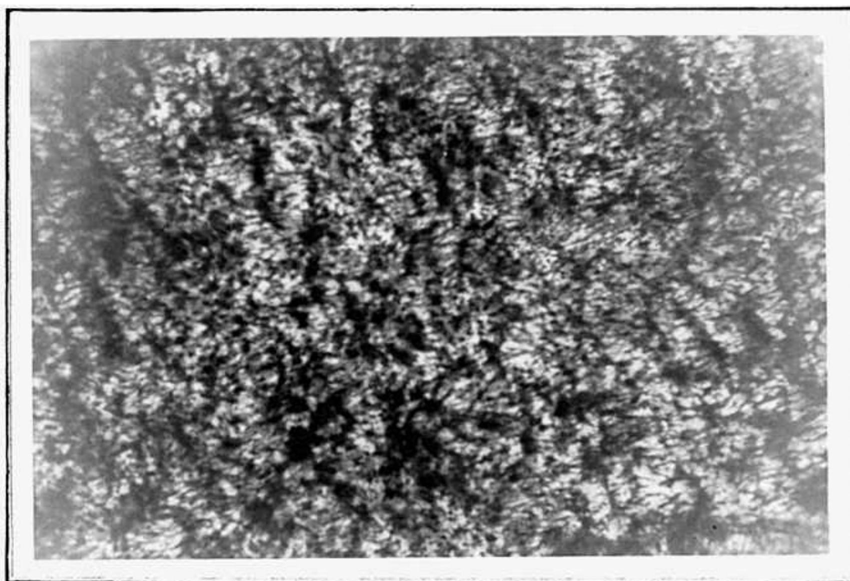

Fig. 9. TEM photographs of LLDPE-*g*-AA membranes and LLDPE-*g*-AA (× 50,000).

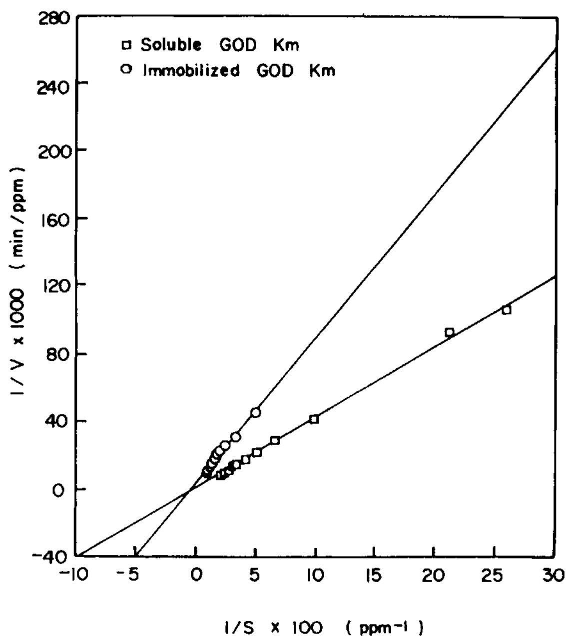

Fig. 10. Lineweaver–Burk plot for soluble and immobilized GOD. The curvature in the plot for the immobilized GOD is $K_m = 0.0017M$, and for the soluble GOD, $K_m = 0.0025M$.

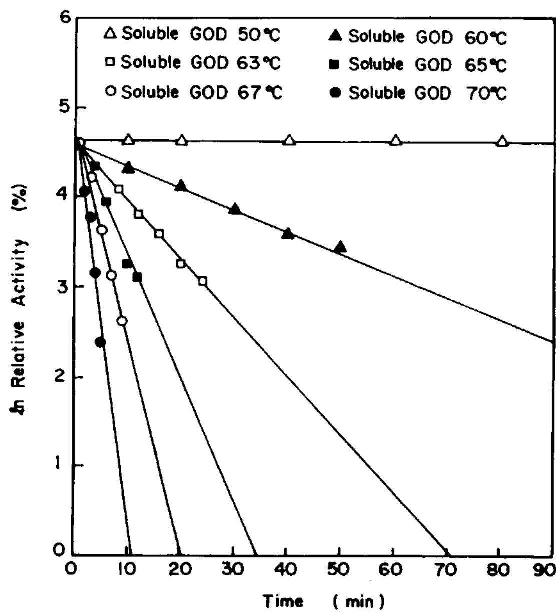

Fig. 11. Activity of the soluble GOD as a function of time at various temperature.

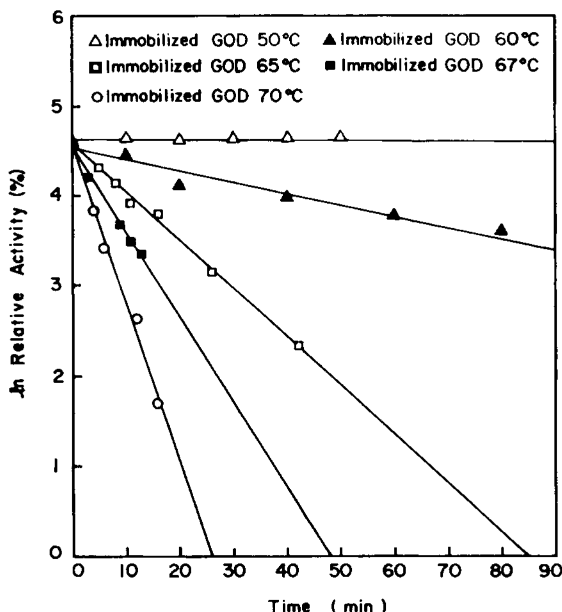

Fig. 12. Activity of the immobilized GOD as a function of time at various temperature.

order behavior is immediately revealed. Both plots were in good agreement with the observations of Malikkides and Weiland[11]; the immobilized enzyme showed reduced sensitivity to thermal deactivation over the soluble form. The rate constants for denaturation of both forms of the enzymes are given in Table I along with the standard deviation of the least-squares fit of the rate data. These values indicated that the temperature dependence of k_d may be of the Arrhenius form, given by the expression

$$k_d = K_0 \exp(-E_a/RT) \tag{1}$$

The rate constants of Table I are plotted in the form suggested by eq. (1) in Figure 13. The Arrhenius kinetics clearly represented the deactivation behavior. The activation energies for denaturation of the immobilized and soluble enzyme were found to be E_a = 60.13 and E_a = 66.35 kcal/mol, respectively. The rate

TABLE I
The k_d of Soluble and Immobilized Glucose Oxidase

Temp (°C)	Soluble GOD (min^{-1})	Immobilized GOD (min^{-1})
60	0.0238	0.0125
63	0.0632	—
65	0.1357	0.0540
67	0.2310	0.0940
70	0.4261	0.1737

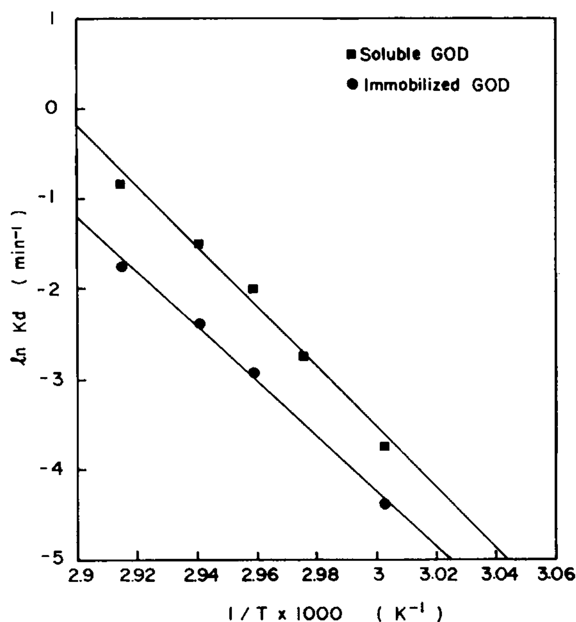

Fig. 13. Relationship between temperature and decay constant (K_d). The curvature in the plot for the immobilized GOD is E_a = 60.13 kcal/mol, and for the soluble GOD, E_a = 66.35 kcal/mol.

at which it was denatured was a function of temperature. These activation energies were much higher than those found in most chemical reaction and they clearly indicated, in quantitative terms, the extreme thermal sensitivity of glucose oxidase.

CONCLUSION

1. The immobilized enzyme is less thermal sensitive than the soluble form. The rate at which it is denatured is a function of temperature.
2. Basis polyethylene, thickness, monomer type, and degree of grafting are the major factors affecting the activity of the glucose oxidase immobilized membrane.
3. The SEM and TEM photographs show PE-*g*-AA to be a homogeneous grafted film.
4. As the degree of grafting increases, the hydrophilicity of the grafted film and the activity increase.

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