Immobilization of Glucoamylase on Polymer Surface by Radiation-Induced Polymerization of Glass-Forming Monomers at Low Temperatures

MASARU YOSHIDA and ISAO KAETSU, *Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Takasaki, Gunma, Japan*

Synopsis

Glucoamylase was immobilized in hydrophilic porous poly(2-hydroxyethyl methacrylate) (PHEMA) and hydrophobic microsphere poly(diethylene glycol dimethacrylate) (PDGDA) by radiation-induced polymerization at low temperatures, in the presence of acetate buffer solution. The distribution on the matrix of immobilized glucoamylase was investigated using fluorescein isothiocyanate (FITC)-conjugated glucoamylase and the fluorescence microscope. It was found that in the porous PHEMA system, the FITC-conjugated glucoamylase is present mainly on the interface between polymer membrane and pore structure and partly in the polymer, while in the microsphere PDGDA system the immobilized glucoamylase is present merely on the surface of the polymer microsphere.

INTRODUCTION

The authors studied the new immobilization method by radiation-induced polymerization of glass-forming monomers at low temperatures.[1-10] This method can be generally applicable to fixation and release of various biocatalysts and biologically active substances such as enzymes, functional proteins, microbial cells, tissue cells, and drugs.

It was deduced that the present immobilized material is characteristic for its surface activity because of the immobilization of biocomponents on the polymer surface. The present method is the immobilization by the principle of physical trapping of biocomponents owing to attachment or adhesion of biocomponent on polymer with polymerization, not by the chemical binding with covalent bonds. The immobilization on the carrier surface by the physical trapping method is little known. The characteristic of surface activity is applicable to various kinds of surface reactions such as enzymatic reactions with solid or high-molecular-weight substrates. However, the surface immobilization mechanism on polymer has not yet been proved clearly. This report concerns the study of the surface immobilization mechanism in the present method, including fluorescence microscopic observation.

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EXPERIMENTAL

Materials

2-Hydroxyethyl methacrylate (HEMA) and diethylene glycol dimethacrylate (DGDA) were obtained from Shin-Nakamura Chemical Co., Ltd., and purified by distillation according to the conventional methods before use.

Aspergillus niger glucoamylase (NOVO Industry A.S., Denmark; 150 NOVO AG-units/ml), maltose (Tokyo Kasei Kogyo Co., Ltd; $[\alpha]_D^{20} = 129.0–130.5$), soluble starch (Katayama Chemical Co., Ltd), and glucose (Kishida Chemical Co., Ltd; $[\alpha]_D^{20} = 52.5–53.0$) were used.

Fluorescein isothiocyanate (FITC) obtained from Aldrich Chemical Co. Inc. and commercially available FITC-conjugated anti-rat IgG (the ratio of optical density at 280/495 is 1.3, and molar ratio F/P is 3.1) obtained from Miles Laboratories Ltd. (U.K.) were used as phosphors.

Preparation of Radiation-Polymerized Composites for Release (Dissolution) Test

In HEMA–buffer (homogeneous mixture) system (Fig. 1), a mixture (1 ml in total volume) of 0.1*M* acetate buffer solution (pH 4.5) containing HEMA (the composition ratio of buffer and HEMA differed with the selected monomer concentration): and 40 mg glucoamylase were charged into an 8-mm-diam glass ampoule and then degassed (10^{-3} mm Hg). The ampoule was cooled rapidly to −78°C (Dry Ice–methanol system) and irradiated at −78°C for 1 hr at a dose rate of 5×10^5 R/hr by gamma rays from a ^{60}Co source. After irradiation, a rodlike composite, 8 mm in diameter and 20 mm long, was obtained. For comparison, low-molecular-weight compounds such as maltose (a substrate for glucoamylase) and glucose (hydrolyzed product) were also entrapped in porous

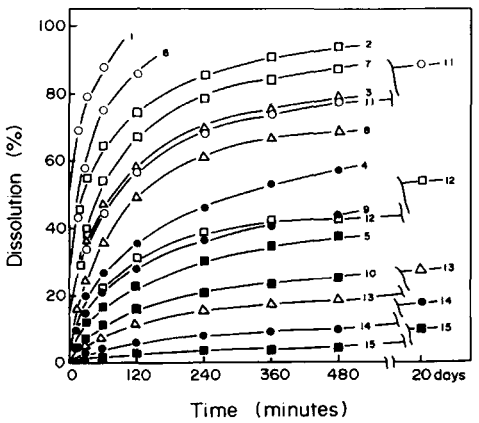


Fig. 1. Relationship between release of immobilized compounds such as glucoamylase, maltose, and glucose from porous PHEMA matrix and the time after start of the test. Immobilized compound: 40 mg; HEMA–buffer (pH 4.5) mixture: 1 ml; irradiation: 5×10^5 R/hr for 1 hr at −78°C *in vacuo*; matrix size: 8 in diameter and 20 mm long. Composition: (○) 10% HEMA–90% buffer; (□) 30% HEMA–70% buffer; (△) 50% HEMA–50% buffer; (●) 70% HEMA–30% buffer; (■) 100% HEMA. Immobilized compound: glucose (samples 1–5); maltose (samples 6–10); glucoamylase (samples 11–15).

PHEMA matrix under the same conditions described above. The composites from 10–40, 50–80, and 90–100% HEMA were spongelike white gel, hard spongelike white gel, and rigid transparent gel, respectively.

In DGDA–buffer (suspension of buffer and hydrophobic monomer) system [Fig. 2(a)], all experiments were carried out by the same methods as in the HEMA–buffer system. The hydrophobic polymerized composite was microsphere or particle at a monomer concentration range lower than about 60%.

The release (dissolution) test was carried out at 45°C by shaking the composite with 500 ml 0.1*M* acetate buffer solution (pH 4.5) as medium. At a selected time interval, 5 ml of the release medium was sampled and assayed spectrophotometrically with a Shimazu double-beam spectrophotometer, model UV-200. That is, glucose was detected by absorption at 505 nm, adding GOD-PODLK (obtained from Nagase Sangyo Co., Ltd.), which consists of glucose oxidase, glucose peroxidase, and chromogen (coloring reagent) and catalyzes the following reactions[11]:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{dye(red)} \xrightarrow{\text{glucose peroxidase}} H_2O + \text{dye(ox)} \quad (2)$$

In the case of maltose, the maltose released from the matrix was completely hydrolyzed with excess glucoamylase (from *Aspergillus niger*,[1] and its amount was estimated from the hydrozlyed product as glucose. The amount of released glucoamylase was assayed spectrophotometrically by measuring optical density at 268 nm.

The release rate (k) of glucoamylase entrapped in a matrix was determined according to Higuchi's equation.[12–17]

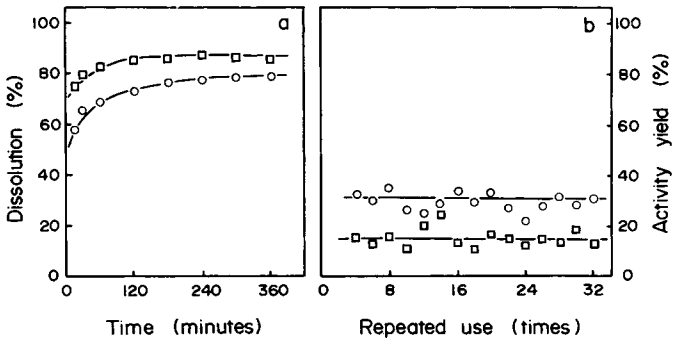


Fig. 2. (a) Relationship between release of glucoamylase from particle-form PDGDA matrix and time start of the test. A mixture of 40 mg glucoamylase and 1 ml DGDA monomer in buffer (pH 4.5) was charged into an 8-mm glass ampoule. The ampoule was degassed. After shaking, the ampoule was irradiated for 1 hr at a dose rate of 5×10^5 R/hr at −78°C *in vacuo*. The release test (leakage test) was carried out at 45°C with buffer (pH 4.5, 1000 ml) as a medium. DGDA monomer concentration: (○) 20%; (□) 50%. (b) Relationship between number of batch enzyme reaction (repeated use) and activity yield of immobilized glucoamylase in particle form. A mixture of 0.8 μg glucoamylase and 1 ml DGDA monomer in buffer (pH 4.5) was charged into an 8-mm glass ampoule. The irradiation conditions were the same as those in (a). Batch enzyme reaction was carried out at 45°C for 1 hr with 10 ml maltose solution (pH 4.5) as substrate. DGDA monomer concentration: (○) 20%; (□) 50%.

Preparation of Immobilized Glucoamylase

The preparations of immobilized glucoamylase for a hydrophobic system such as DGDA monomer and a hydrophilic system such as HEMA monomer in Figures 2(a) and 3 were carried out as follows: Glucoamylase, 0.8 μg, was dissolved in 0.1*M* acetate buffer solution (pH 4.5), and then a monomer was added to form a 1-ml mixture total volume. The ratio of buffer and monomer in this total volume changed according to the selected monomer concentration. The monomer concentration [M] is given as

$$[\mathrm{M}]\ (\%) = \frac{\text{monomer (ml)}}{\text{monomer (ml)} + \text{buffer (ml)}} \times 100 \qquad (3)$$

The mixture was charged into an 8-mm-diameter glass ampoule and degassed (10^{-3} mm Hg). In the HEMA system, the whole mixture dissolved homogeneously in the required composition except for the 100% HEMA composition. However, in the hydrophobic monomer system, no DGDA dissolved in the buffer containing enzyme. Therefore, this mixture charged in a glass ampoule was quickly shaken. Immediately after shaking, the ampoule was frozen at a low temperature (−78°C).

In all cases, the irradiation was carried out at −78°C for 1 hr at a dose rate of 5×10^5 R/hr *in vacuo*. After irradiation, the immobilized enzyme was cut into 10 samples 8 mm in diameter and 2 mm long and used for leakage test and activity assay in the case of hydrophilic composite. The composite obtained from hydrophobic monomer such as DGDA had a microspheric form. This enzyme–polymer composite was used for leakage tests and activity assays as polymerized form.

The activity yield of the immobilized enzyme was assayed as follows: The immobilized enzyme composite made under the above conditions was added to 1% maltose solution (pH 4.5) as a substrate in an ampoule and used for enzyme batch reaction. The enzyme batch reaction was carried out for all systems by

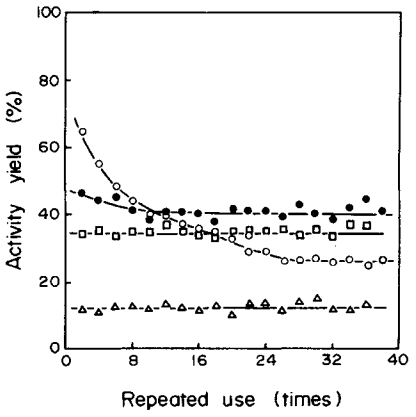


Fig. 3. Relationship between number of batch enzyme reaction (repeated use) and activity yield of immobilized glucoamylase in porous PHEMA matrix. A mixture of 0.8 μg glucoamylase and 1 ml HEMA monomer in buffer (pH 4.5) was charged into an 8-mm glass ampoule. The ampoule was sealed off under a vacuum of 10^{-3} mm Hg and then irradiated for 1 hr at a dose rate of 5×10^5 R/hr at −78°C *in vacuo*. Batch enzyme reaction was carried out at 45°C for 1 hr with 10 ml 1% maltose solution (pH 4.5). HEMA monomer concentration: (○) 30%; (●) 40%; (□) 50%; (△) 70%.

shaking the ampoule for 1 hr at 45°C. After reaction, the glucose as hydrolyzed product was assayed spectrophotometrically at 505 nm, adding GOD-PODLK (from Nagase Sangyo Co., Ltd.), which consist of glucose oxidase, glucose peroxidase, and chromogen.[11]

The activity yield of the immobilized enzyme was expressed as follows:

$$\text{activity yield (\%)} = \frac{I_a}{N_a} \times 100 \tag{4}$$

where I_a is the activity of immobilized glucoamylase for each enzyme batch reaction and N_a is the activity of native glucoamylase in buffer solution of the same quantity as used for the immobilization.

Fluorescence Measurement

The preparation of FITC-conjugated glucoamylase was carried out as follows: FITC (0.5 mg) was dissolved in 0.25 ml 0.1*M* carbonate buffer (pH 9.0), and then 1 ml 0.1*M* carbonate buffer (pH 9.0) containing 10 mg glucoamylase from *Aspergillus niger* was mixed with the solution. The mixture was shaken at 4°C for 10 hr. In this case, free FITC or free glucoamylase in the reaction system was not removed.

A mixture of 1 ml glass-forming monomer (HEMA or DGDA) and reaction mixtures containing FITC-conjugated glucoamylase was charged into an 8-mm-diam glass ampoule. The immobilization was carried out according to the same methods as in the immobilization of glucoamylase. The immobilized polymer–enzyme composites, containing FITC-conjugated glucoamylase (the porous PHEMA composite cut to a 15–25 μm slice membrane and the PDGDA composite a 20- to 30-μm microsphere), were washed by shaking at 45°C for one week with excess 0.1*M* carbonate buffer (pH 9.0). The resulting free enzyme or FITC was removed from the matrix. Then, the FITC-conjugated glucoamylase immobilized firmly on the matrix was observed with a fluorescence microscope (Nippon Kogaku Co., Ltd., model FT).

The effect of γ irradiation on FITC itself was examined. It was found that under the irradiation at low temperatures, the activity damage of fluorescence was very little.

The fluorescence-microscopic observation was done also on the immobilized composite containing commercial FITC-conjugated anti-rat IgG for comparison. In this case, no free FITC was present in FITC-conjugated anti-rat IgG solution.

Dialysis Measurement

The relative dialysis rates, that is, relative diffusion rates (D), of glucoamylase, maltose, and glucose were determined for porous PHEMA films using a dialysis cell.[18,19] Rate studies were carried out at 45°C by placing the whole dialysis assembly in a constant-temperature bath. Thirty-eight milliliters of the dialysis solution (prepared by dissolving 10 g solute in 100 ml buffer, pH 4.5) was placed inside the cell, and 500 ml buffer (pH 4.5) was placed in the modified cylindrical flask outside the cell.

At a selected time interval, the amounts of glucose, maltose, and glucoamylase

that diffused out through porous PHEMA film were measured by the methods already described above.

RESULTS AND DISCUSSION

Release Property of Immobilized Glucoamylase

As previously studied,[1] the porous polymer–enzyme composite formed in the polymerization of hydrophilic monomer (HEMA)–buffer solution mixture including enzyme at low temperatures. The reason for this pore structure was attributed to the space volume of ice crystallized at low temperatures. The used monomer, a glass-forming monomer, took a supercooled state at low temperatures, and polymerized dispersing the ice. Therefore, the pore formed in the polymer matrix after polymerization. It was found that the porosity in the matrix changed with the composition of monomer and buffer, in other words, with the monomer concentration. In the case of very porous matrix obtained under low monomer concentration, leakage of enzyme occurred. However, this enzyme leakage has not been proved entirely. The release (leakage) of immobilized glucoamylase from the polymer matrices was then studied by means of solution analysis.

The release profile of glucoamylase from the porous PHEMA matrix is shown in Figure 1 as a function of HEMA concentration in buffer solution. According to this result, the release rate obviously depended on the monomer concentration. The release rate of immobilized enzyme decreased with increasing HEMA concentration in all systems. On the other hand,a microspheric polymer was obtained in the radiation polymerization of hydrophobic monomer (such as DGDA)–buffer mixture at low temperatures.[20]

The release profile of glucoamylase from DGDA sphere matrix was studied as shown in Figure 2(a). The dissolution from hydrophobic polymer showed very high values even at the initial stage of release as shown in Figure 2(a). This suggests that the enzyme loss occurred by washing after immobilization, that is, before the release experiment owing to the enzyme being isolated and remaining in the water phase. This enzyme loss decreases with increasing the overall (total) surface area of hydrophobic particles present in the system. In other words, the activity yield increases with increase in the total surface area of particles. The average sphere diameter formed increased with increasing monomer concentration. The same tendency was observed in other hydrophobic monomer systems.[21] The average sizes of the particle at 20 and 50% DGDA estimated from microscopic observation were 50 and 180 μm, respectively, while the average numbers of particle at 20 and 50% DGDA calculated from the above average size and amount of polymer in the system were 3.06×10^6 and 1.63×10^5 particles, respectively. Therefore, the apparent total surface area (as the summation of particle number and individual surface area of a particle) is 240 and 170 cm^2 for the 20 and 50% DGDA systems, respectively.

According to the results of Figure 2(a), the amount of loss in the 50% DGDA system was larger than that in the 20% DGDA system, while activity yield in the 50% DGDA system was smaller than that in the 20% DGDA system. This difference can be attributed to the difference in total surface area of the particles for the reasons described above.

Activity Yield of Immobilized Glucoamylase

The enzyme reaction of immobilized glucoamylase was carried out repeatedly in a batch reaction using maltose solution (pH 4.5) as a substrate. The results are shown in Figures 2(b) and 3.

In the previous study, in porous hydrophilic PHEMA system, a decrease in apparent activity was observed with repeated use in the immobilized systems at monomer concentrations below 50%.[1] However, as shown in Figure 3, the activity yield became constant after extremely long uses to take a certain saturated value even in the 30% HEMA system. As shown in Figure 1, about 43% glucoamylase leaks out considerably from porous matrix in the 30% HEMA system after 480 min, while leakage of glucoamylase is only 10% after 480 min in the 70% HEMA system. From these results, it is certain that the decrease of activity from 75% (initial value) to 26% (after 40 times of use) can be attributed mainly to the leakage of enzyme from porous matrix in the 30% HEMA system (Fig. 3).

On the other hand, Figure 2(b) shows that the activity is not changed much with long use in the hydrophobic PDGDA system, even at low monomer concentration such as 20%, though the initial activity is rather low. This result agreed with the result in Figure 2(a) that initial loss of enzyme by dissolution is large but increase in dissolution with time is small. This means that the leakage of glucoamylase is small in hydrophobic sphere systems. In the case of hydrophobic sphere systems (Fig. 2), the amount of undissolved enzyme is 15–25% [Fig. 2(a)], while the amount of fixed active enzyme obtained from activity yield is 15–35% [Fig. 2(b)]. This fact means that all unleaked (undissolved) enzymes are contributed to the activity. On the other hand, in hydrophilic porous polymer systems, the amount of unleaked enzyme is 46% after 20 days (Fig. 1), and the amount of active enzyme is only about 35% in the 30% HEMA system.

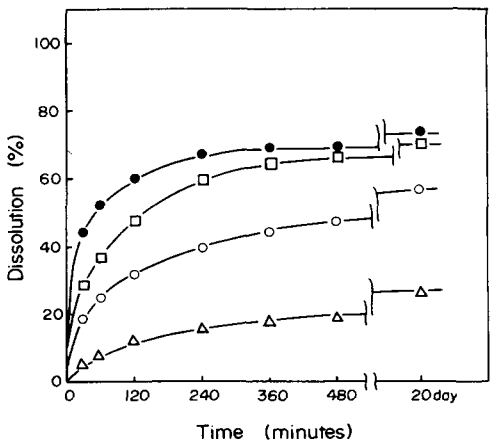


Fig 4. Relationship between the release of glucoamylase from porous PHEMA matrix having various surface areas in 50% HEMA–50% buffer (pH 4.5) system and the time after start of the test. A mixture of 40 mg glucoamylase and 1 ml 50% HEMA monomer in buffer (pH 4.5) was charged into an 8-mm glass ampoule. The other experimental conditions were the same as those in Fig. 1. Surface area (cm^2): (△) 6; (○) 25; (□) 60; (●) 400.

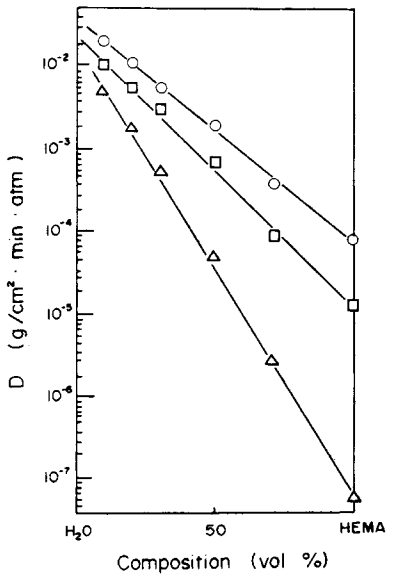


Fig. 5. Relationship between diffusion rate (D) of glucose, maltose, and glucoamylase and water–HEMA composition. PHEMA membrane thickness: 400 μm. Solute: (○) glucose; (□) maltose; (△) glucoamylase.

The unleaked enzyme is 82% and the active enzyme is only 12% in the 70% HEMA system. This means that the unleaked enzymes of 11 and 70% hardly contributed to activity yield in 30 and 70% HEMA systems. It is natural to deduce that this unleaked and uncontributed enzyme in hydrophilic polymer is occluded inside the matrix, while no enzyme is occluded inside the polymer matrix in the case of hydrophobic polymer.

To clarify this point, the effect of surface area of matrix on the leakage and activity of immobilized glucoamylase was studied by cutting a rod polymer matrix (8 mm in diameter and 20 mm long at 50% HEMA into the slice pieces). The results are shown in Figure 4. The release rate and the saturated dissolution amount of glucoamylase increased with increasing surface area of the matrix. According to this result, the leaked glucoamylase reached about 65–70%, and the final unleaked enzyme was less than 30% by cutting to a 400 cm^2 surface area. This value is comparable to the constant activity yield (saturated yield is 33%) in Figure 3. All unleaked enzyme contributed to the activity yield in this case. However, in higher HEMA concentration systems, more than 50%, the amount of inactive and occluded enzyme inside the matrix was relatively large and remained so even after fine cutting. The content of active, unleaked enzyme increased with cutting, but the leaked enzyme increased also with cutting. These results show that the activity strongly depends on the pore structure (monomer concentration in the polymerization) and surface area in the polymer. This fact supports the notion that the active, but, unleaked enzyme is distributed firmly on the surface of the pores and the reaction is carried out on the pore structure, not inside the matrix.

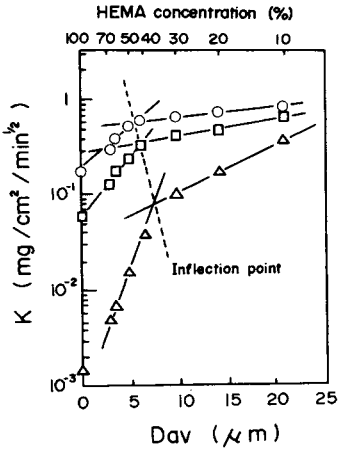


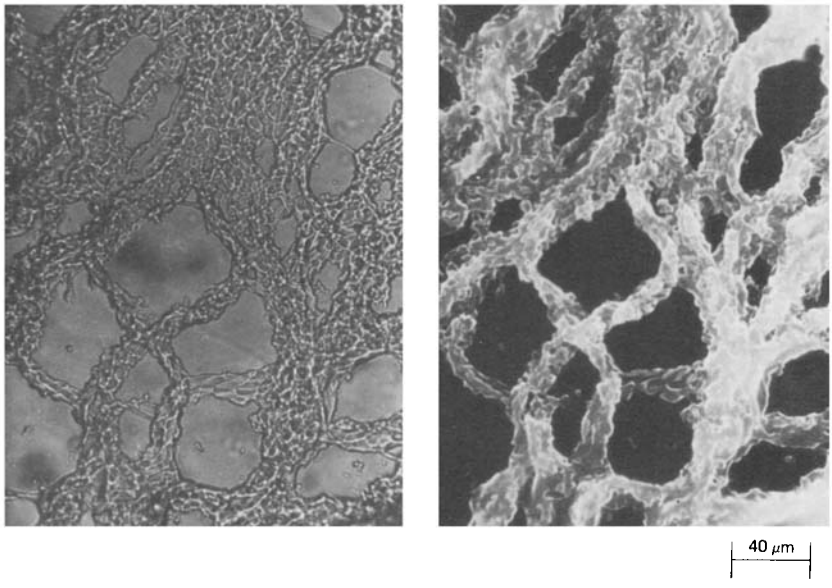
Fig. 6. Relationship between magnitude (k) of leakage profile of immobilized compounds and average diameter of pore (or HEMA concentration). The experimental conditions were the same as those in Fig. 1. Immobilized compound: (○) glucose; (□) maltose; (△) glucoamylase.

Another Proof for the Surface Immobilization Mechanism

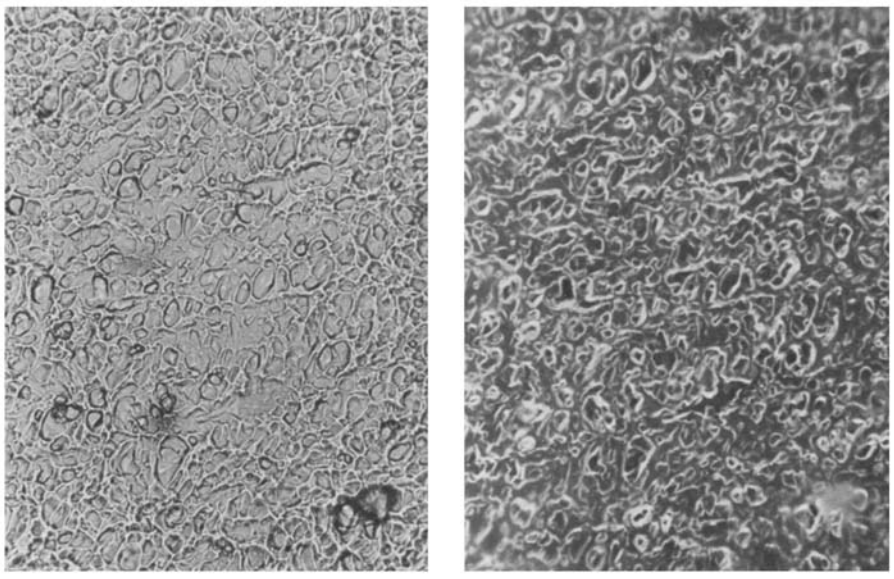
Figure 5 shows the diffusion profiles of various substrates having different molecular weights through the polymer matrices prepared under various HEMA concentrations in water. According to this result, the diffusion rate (D) of the substrate decreased remarkably with increasing monomer concentration, that is, with decreasing porosity in the matrix. The diffusion rate at 10% monomer concentration is more than 10^3 times greater than that at 100% monomer concentration (nonporous membrane) owing to the easy diffusion through the pore. The diffusion at 100% polymer matrix corresponds to that in the nonporous polymer membrane in a swelling state. Therefore, from this result it is certain that the substrate diffuses mainly through the pore and reacts on the pore surface with the immobilized enzyme in the porous systems other than the 100% HEMA matrix.

On the other hand, the release rate constant (k) for the release of drug from matrices[13] was plotted against the HEMA concentration and the average pore diameter of the matrices (D_{av}). The k value was evaluated according to Higuchi's equation for the release and obtained from the data in Figure 1. The result is shown in Figure 6. The k value showed an inflection point at a certain D_{av} and HEMA concentration. This means that the release (dissolution out or leakage in another expression) of these substances is accelerated or retarded suddenly at a certain porosity (or monomer concentration) at this point. The diffusion in the matrix becomes easy in larger pore regions and becomes difficult in smaller pore regions than that of the inflection point. The inflection point obviously differs with the molecular weight of the substances. It is reasonable that enzyme took the point at a larger pore diameter (at a lower monomer concentration) than in low-molecular-weight substrate.

The results in Figure 6 shows that the diffusion of the substrate became difficult at about 40–50% monomer concentration. Therefore, there is an optimum monomer concentration range between 30 and 50% as a result of no enzyme leakage and easy substrate diffusion. It is noted that this range between the two

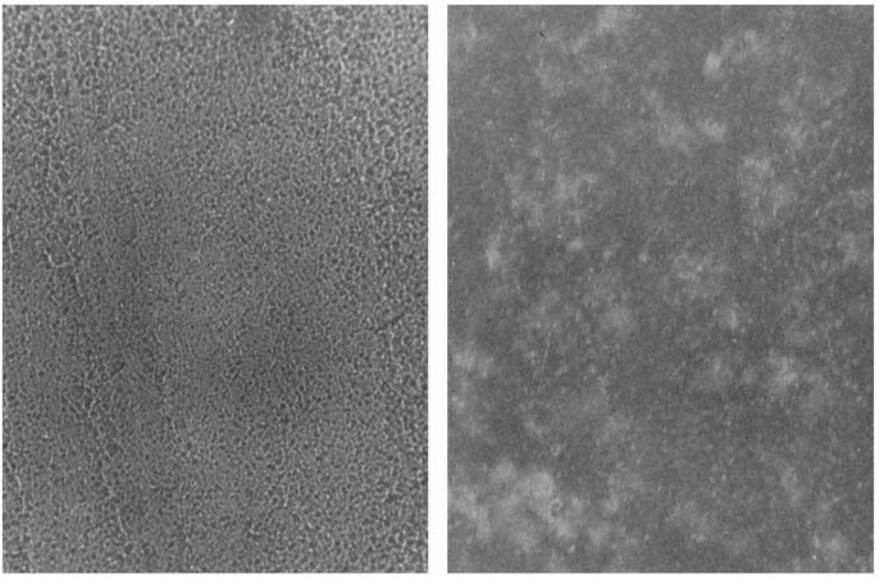


(a) (aa)



(b) (bb)

Fig. 7. Fluorescence microphotographs of various porous PHEMA matrices containing FITC conjugated glucoamylase. Nonfluorescence microphotograph: (a), (b), and (c). Fluorescence microphotograph: (aa), (bb), and (cc). HEMA concentration: 10% (a) and (aa); 50% (b) and (bb); 80% (c) and (cc).

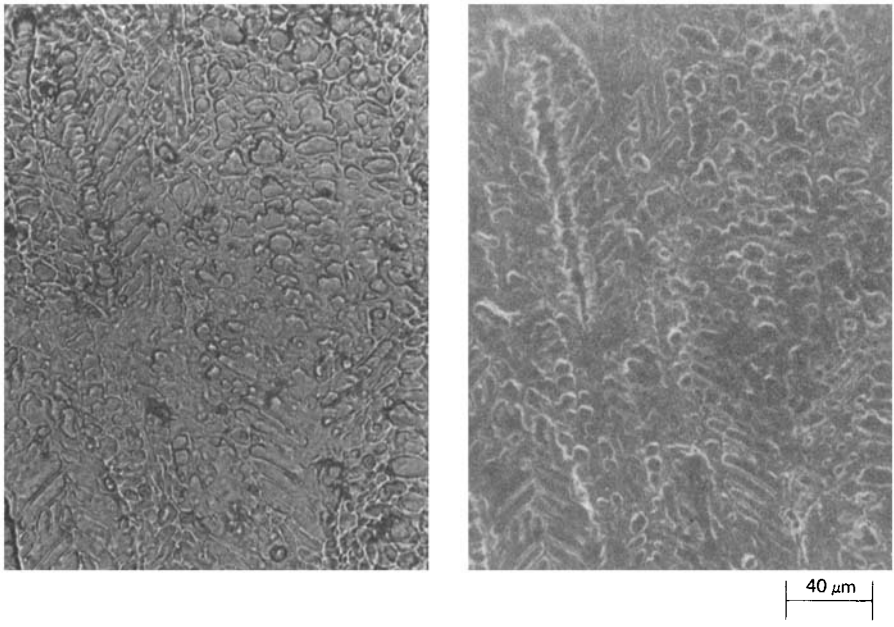


(c) (cc)

Fig. 7. (*Continued from previous page.*)

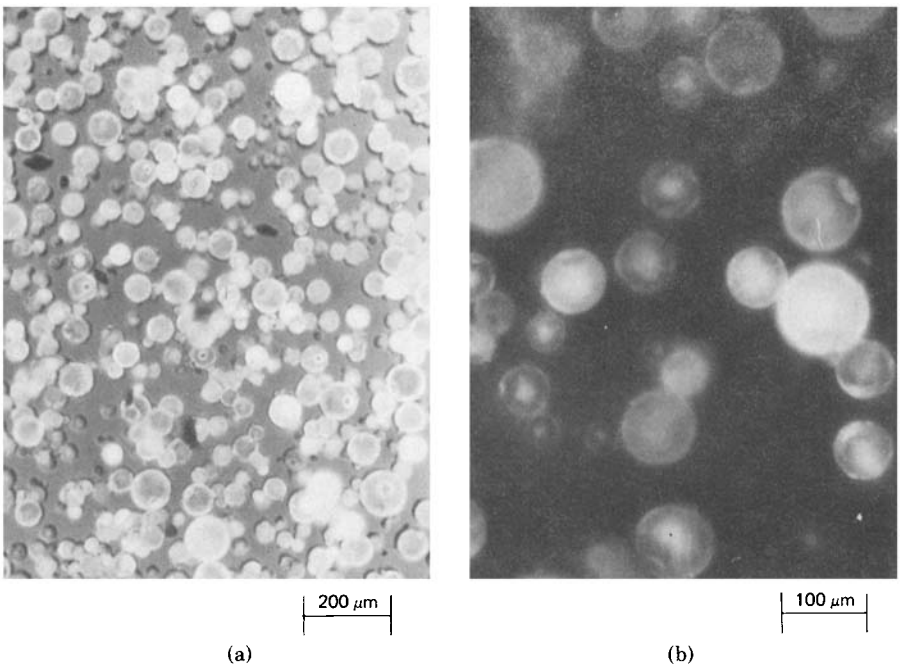
inflection points in Figure 6 agree with the monomer concentration range of maximum activity yields in the monomer concentration dependence of activity.[1] It is probable that the reaction on the pore surface is the main process in the present immobilized enzyme, judging from the important effect of substrate diffusion through the pore. The fact of effective reaction with hydrophobic polymer carrier supports the surface reaction, because substrate can hardly penetrate into the hydrophobic matrix. Furthermore, the enzymatic reactions were carried out by immobilized glucoamylase using 1% soluble starch solution as a high-molecular-weight substrate and also maltose solution having a different molecular weight. The activity yields for both substrate were comparable in the immobilized system with hydrophobic polymer and with relatively lower concentration of the hydrophilic system.[23] This fact suggests that the enzyme reaction between the immobilized enzyme and the substrate was carried out on the surface of the polymer in these systems.

As already reported,[21,22] the K_m value in the Michaelis–Menten coefficient in the immobilized enzyme or microbial cell in the above conditions was near that of native enzyme or intact cells. The fact also supports the surface immobilization. Furthermore, the hydrolysis reaction with the solid and high molecular weight substrate such as cellulose, cellulosic waste, and various proteins was carried out effectively by the immobilized hydrolases. Antigen was also caught effectively by immobilized antibody such as globulin.[10] These results can be explained only by surface reactions. Moreover, the authors tried to obtained direct proof for surface immobilization by means of the fluorescence method (see next section).



(a) (b)

Fig. 8. Fluorescence microphotographs of porous PHEMA matrix (50% HEMA monomer concentration) containing FITC-conjugated anti-rat IgG: (a) nonfluorescence microphotograph; (b) fluorescence microphotograph.



(a) (b)

Fig. 9. Fluorescence microphotographs of microparticle PDGDA matrix containing FITC-conjugated glucoamylase. DGDA concentration: 20% DGDA in buffer (pH 4.5).

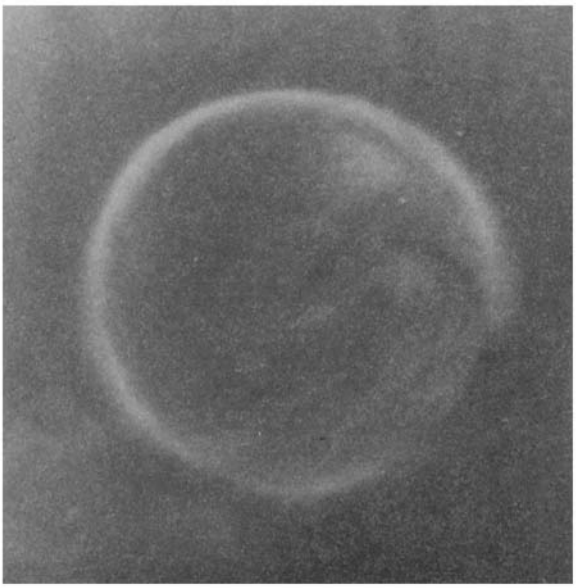


Fig. 10. Fluorescence microphotograph of a slice of microparticle PDGDA matrix containing FITC-conjugated glucoamylase. A microparticle PDGDA matrix, in Fig. 9, was cut into round slices. A microparticle slice was then observed under a fluorescence microscope. The experimental conditions were the same as those in Fig. 9.

Fluorescence Observation

The distribution in the matrix of immobilized glucoamylase was investigated using FITC-conjugated glucoamylase and fluorescence microphotograph. The results are shown in Figures 7–10. After the removal of free FITC and freely isolated FITC–enzyme in the matrix by washing, the FITC-conjugated glucoamylase which was firmly immobilized on the matrix was observed under a fluorescence microscope. For comparison, only FITC was immobilized in the matrix by the same polymerization under the experimental conditions as described in Figures 7 and 8. In the latter, the fluorescence can barely be observed with the fluorescence microscope after washing with excess carbonate buffer (pH 9.0).

It was found that in the case of porous PHEMA system, the FITC-conjugated glucoamylase was observed mainly on the interface between polymer membrane and pore structure and partly in the polymer matrix, as shown in Figure 7. Commercially available FITC-conjugated anti-rat IgG was used for a comparison. The distribution of the FITC-conjugated anti-rat IgG in porous matrix in 50% HEMA polymer system [Fig. 8(b)] was very similar to that of the FITC-conjugated glucoamylase in 50% HEMA system as shown in Figure 7(bb). Moreover, the porous structure observed by the fluorescence microscope clearly agreed with that observed by an optical microscope.

Figure 9 shows that in the PDGDA microparticle matrix, the intensity of the fluorescence differs remarkably from that of the particle. On the other hand, the fluorescence distribution in a cross section of microparticles was obtained by cutting the sphere polymer to circular slices, as shown in Figure 10. According

to Figure 10, the fluorescence can be observed only on the surface of the particle. Consequently, it can be said that in hydrophobic particle systems, the firmly immobilized enzyme is present merely on the surface of the matrix.

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