Immobilization of enzymes by radiation-induced polymerization of glass-forming monomers: 2. Effects of cooling rate and solvent on porosity and activity of immobilized enzymes

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The effect of cooling rate of a monomeric system on the porosity and activity of an immobilized enzyme prepared by radiation-induced polymerization of 2-hydroxyethyl methacrylate at low temperatures has been studied. Slow cooling gave the same effect on porosity of the polymer as decreasing the monomer concentration. A glass-forming solvent such as diethylene glycol was added to water to study the effect of the supercooling tendency of the solvent. Addition of diethylene glycol decreased porosity and also enzymic activity. Water was replaced by the miscible solvent *p*-dioxane and the immiscible solvent n-decane in order to clarify the effect of solvent. *p*-Dioxane had a similar effect to water on the relation between the monomer concentration, porosity and activity. On the other hand, polymer prepared from the system containing n-decane showed different immobilization properties owing to the presence of independent pores in the matrix.

INTRODUCTION

We have studied the radiation-induced polymerization of glass-forming monomers and its application to the entrapping of biologically active substances[1–4]. It was found that effective immobilization of the enzyme was possible by polymerizing a mixture of a glass-forming monomer such as 2-hydroxyethyl methacrylate and an aqueous solution of enzyme at low temperatures. The chief characteristic of the polymer formed by this method was the porous structure of the polymer matrix which arose owing to ice dispersed in the supercooled monomer matrix. It was shown that the porous structure gave rise to important effects in activity yield and the variation of activity yield with repeated use of the immobilized enzyme. The porosity can be controlled by changing the combination and composition of the crystallizing (water or organic solvent) and supercooling (monomer) components as well as the cooling rate (crystallization). In this report, the effects of these important factors on the porosity and the activity yield are investigated.

MATERIALS AND METHODS

Glucoamylase, 2-hydroxyethyl methacrylate (HEMA) and maltose used in this work were the same as used in the previous study[4]. Organic solvents such as n-decane, *p*-dioxane, and diethylene glycol (DG) were purified by distillation before use.

Immobilization with the system containing n-decane or *p*-dioxane as a solvent was carried out as follows. Solvent and HEMA were mixed to the required composition. The mixture (homogeneous in the case of *p*-dioxane and heterogeneous in the case of n-decane) was charged into an 8 mm diam. glass ampoule and 0.8 μg of enzyme (2 μl in acetate buffer, pH 4.5) was added to the mixture. The ampoule was sealed off under a vacuum of 10^{-3} mmHg. The sealed ampoule was shaken and then immersed in a Dewar vessel kept at −78°C in dry ice–ethanol. The enzyme was adequately suspended in these systems by shaking. γ-irradiation was carried out using a ^{60}Co source at −78°C for 1 h at a dose rate of 5×10^5 rad/h.

Immobilization with the system containing the DG–buffer solution as mixed solvent was carried out as follows. The enzyme (0.8 μg) was dissolved in 0.1 M acetate buffer (pH 4.5). DG was mixed with the above solution to a total volume of 0.5 ml. 0.5 ml of HEMA was then added and the whole mixture dissolved homogeneously. This mixture was charged into an 8 mm diam. glass ampoule and sealed off under a vacuum of 10^{-3} mmHg. Irradiation was carried out for 1 h at a dose rate of 5×10^5 rad/h, at −78°C. The composite formed in this study was cut into 10 samples 8 mm diam. × 2 mm long and used for enzyme reactions without drying treatment.

The enzyme reaction was carried out by shaking enzyme preparations with a mixture of 5 ml of 1% maltose solution and immobilized glucoamylase at 45°C for 30 min.

The glass transition temperature (T_g) and melting point (T_m) of the monomer–solvent system were determined by differential thermal analysis (d.t.a.).

The parameters describing the porous structure, such as average pore diameter, number of pores/cm^2 and porosity of the composite were determined by optical microscopy. The water content was measured as follows. The polymerized composite was immersed in water at 35°C in order to reach swelling equilibrium and the weight was then determined[4].

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RESULTS AND DISCUSSION

Effect of cooling rate on porosity and enzymatic activity

The pore structure depends on the space occupied by the crystallized component. The cooling rate might be one of the important factors affecting the porosity, because the crystal size of ice varies with the crystallization rate. The effect of cooling rate on pore factors such as average pore diameter, number of pores, porosity and water content are shown in *Figure 1*. According to this result, the average pore diameter increases but the number of pores decreases with a decrease in the cooling rate. Apparent porosity increases with a decrease in cooling rate. This means that porosity can be controlled to some degree by the cooling rate; slow cooling increases the size of the crystal and gives the same effect on pore structure as a decrease in monomer concentration. It is reasonable that the activity yield is larger in the early stages of repeated use of the immobilized enzyme but becomes smaller in the later stages of repeated use, owing to enzyme leakage. This trend is more marked for immobilized enzymes as the cooling rate used in the pretreatment step decreases.

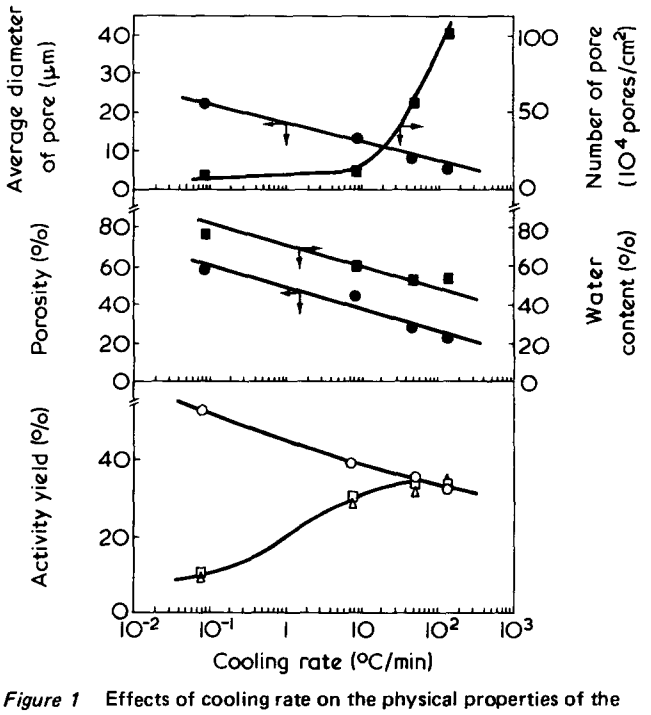

Figure 1 Effects of cooling rate on the physical properties of the pore structure in polymerized composite and on the activity yield of immobilized glucoamylase. HEMA solution containing enzyme was cooled at various rates from 25° to −78°C, and the immobilization was carried out under 5 x 10^5 rad at −78°C, *in vacuo*. Number of batch reactions; ○, 1; □, 5; △, 20

Effect of increasing the supercooling property of the solvent on porosity and activity yield

To investigate the effect of the supercooling of the crystallizing component (solvent), various amounts of DG as a glass-forming solvent were added to water. D.t.a. curves, as shown in *Figures 2a–2c*, showed that the supercooling increased with addition of DG. The porosity obtained and activity yield are shown in *Figure 3* as a function of the water–DG composition. According to these results, the average pore diameter, number of pores, and porosity decreased when the supercooling component, DG, was increased. But the activity yield showed a maximum at a cer-

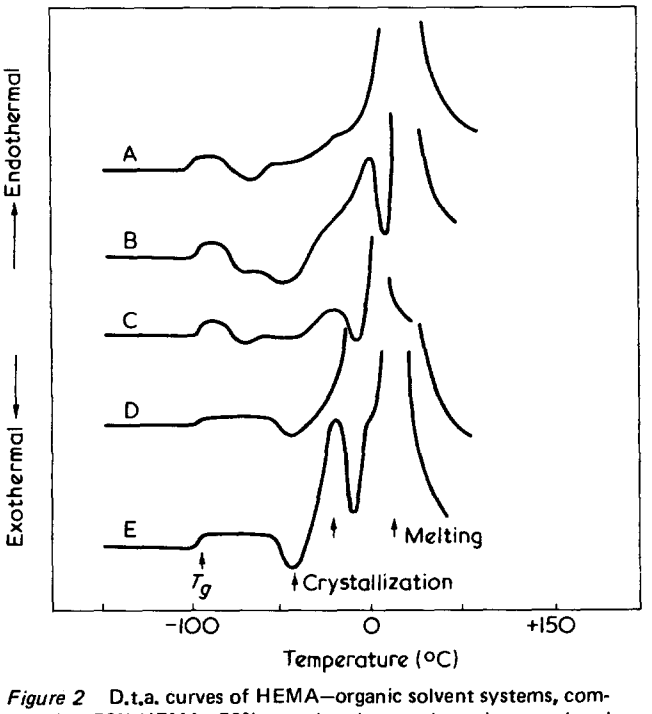

Figure 2 D.t.a. curves of HEMA–organic solvent systems, composed of 50% HEMA–50% organic solvent, where the organic solvent is: A, water; B, 70% water–30% DG; C, DG; D, *p*-dioxane; E, n-decane

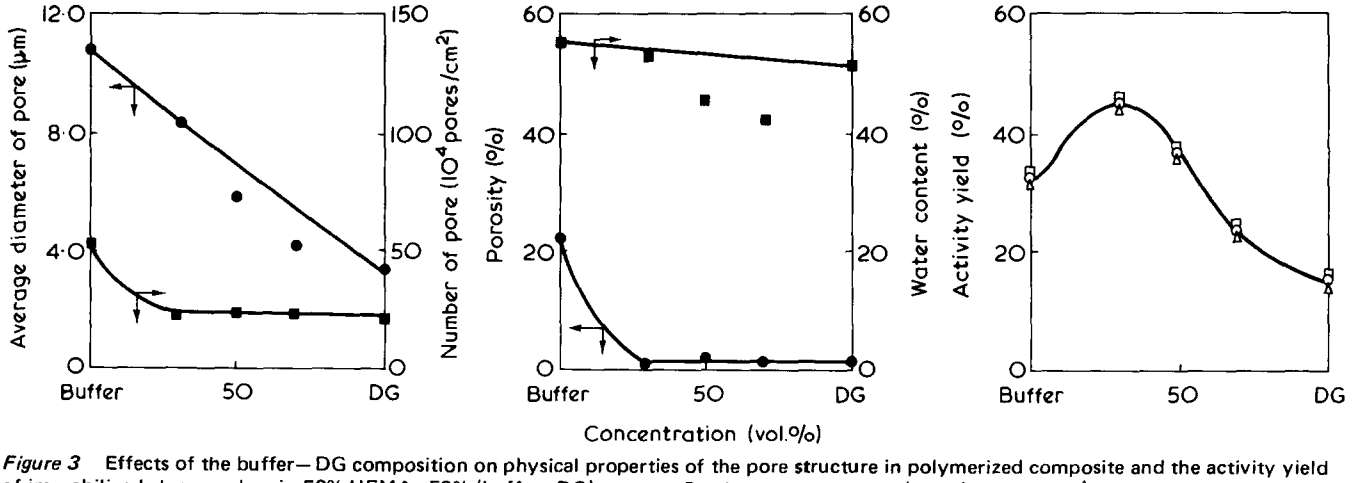

Figure 3 Effects of the buffer–DG composition on physical properties of the pore structure in polymerized composite and the activity yield of immobilized glucoamylase in 50% HEMA–50% (buffer–DG) system. Batch reaction number (times): ○, 1; □, 5; △, 15

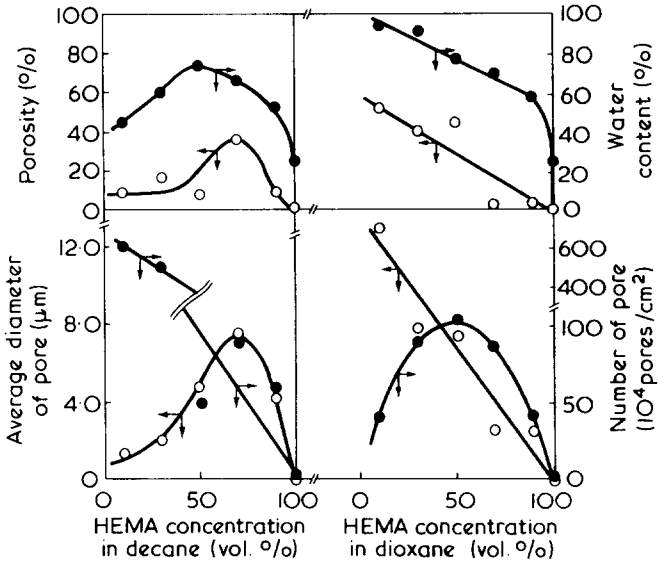

Figure 4 Effects of the HEMA concentration in various organic solvents on the physical properties of the pore structure in polymerized composite

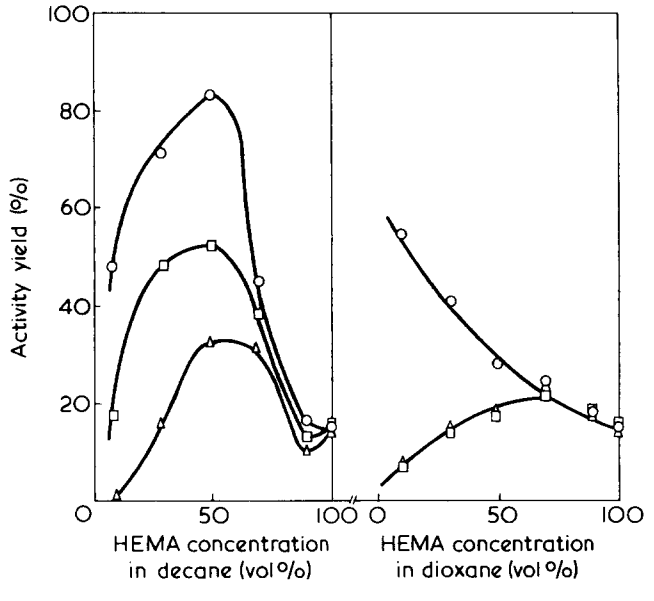

Figure 5 Effects of the HEMA concentration in various organic solvents on the activity yield of immobilized glucoamylase. Batch reaction number (times); (○), 1; (□), 5; (△), 15

tain solvent composition. The monomer concentration used for solvent was the concentration at which enzyme leakage hardly occurred with repeated use. So the dependence of activity on the water–DG composition might be due to the difference in the quantity of enzyme initially entrapped. The enzyme loss due to non-entrapment might increase with increasing water content due to an increase in pore diameter. On the other hand, enzyme buried or occluded inside the matrix and not contributing to the enzyme reaction increases with an increase in supercooling of DG. This is probably the reason for the optimum in activity.

Effect of miscible and immiscible solvents on porosity and enzymatic activity

In order to study the effect of a crystallizing solvent on porosity and on the activity of the immobilized enzyme, water was replaced by other crystallizing solvents. n-Decane and *p*-dioxane were used as examples of miscible and immiscible crystallizing components for the monomer. D.t.a. curves in these systems showed that these solvents completely crystallized at low temperatures, as shown in *Figures 2d* and *2e*.

The relation between monomer–solvent composition and pore factors are shown in *Figure 4*. The effect of pore factors on activity yield in all systems is shown in *Figure 5*.

According to *Figure 4*, porosity exhibited a maximum at a certain HEMA concentration in the n-decane system in contrast to the water[4] or *p*-dioxane system. This result showed that in the n-decane (immiscible solvent) system, much larger numbers of smaller suspended particles formed at low monomer concentration in contrast to the miscible solvent systems in which pore diameter increases due to the joining of pores. According to *Figure 5*, in the n-decane system, a decrease in activity caused by enzyme leakage with repeated use of the immobilized enzyme was observed at high monomer concentration in spite of the smaller porosity, in contrast to the miscible solvent including system. This fact suggests that in the n-decane system, freely isolated enzymes are also present in pores – even in small diameter pores of small porosity composition. The reason might be that in immiscible systems, crystalline enzymes are also dispersed uniformly in the small solvent particle phase as well as in the monomer phase.

On the other hand, in the miscible system, enzyme molecules or enzyme crystals are first dispersed in the homogeneous mixture of monomer and solvent and then, as the solvent crystallization proceeds, partly isolated from the monomer phase in contact with the crystallized solvent. However, the probability of such an isolation of the enzyme decreases with an increase in monomer concentration. This might explain the leakage of enzyme that occurs in the immiscible solvent system but hardly at all in the miscible solvent system in the high monomer concentration region. The result in *Figure 6* shows that enzyme leakage continues much longer with repeated use in the n-decane system than in the *p*-dioxane system. This might be due to the fact that in the

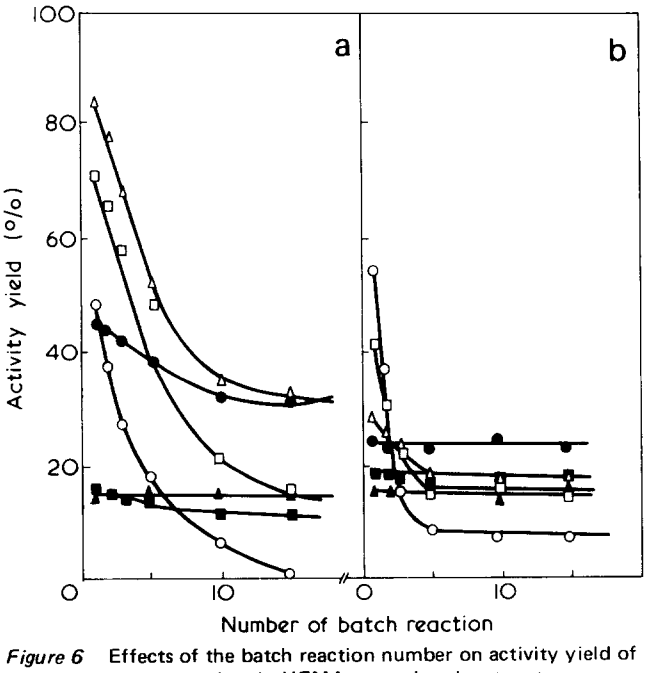

Figure 6 Effects of the batch reaction number on activity yield of immobilized glucoamylase in HEMA–organic solvent systems (a) HEMA–decane; (b) HEMA–dioxane. HEMA concentration: ○, 10%; □, 30%; △, 50%; ●, 70%; ■, 100%

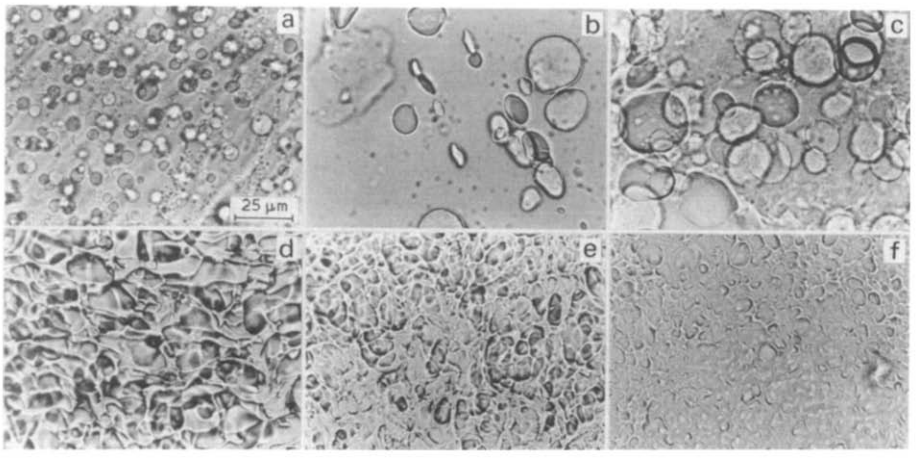

Figure 7 Optical micrographs of pore structure in polymerized composite in the presence of various organic solvents. A, 30% HEMA–70% n-decane; B, 50% HEMA–50% n-decane; C, 70% HEMA–30% n-decane; D, 30% HEMA–70% *p*-dioxane; E, 50% HEMA–50% *p*-dioxane; F, 70% HEMA–30% *p*-dioxane

immiscible solvent including system, the pore had more of the properties of a completely independent cell and the diffusion out of free isolated enzyme takes a longer time than in the *p*-dioxane system. The behaviour and properties of the *p*-dioxane system showed the same tendency as in the water system and the miscible systems, although there was a difference between the two systems with respect to enzyme solubility; that is, the difference between homogeneous dissolved enzyme or uniformly dispersed enzyme crystals. By contrast the immiscible solvent system showed completely different characteristics from the miscible solvent systems. However, it was clear that porosity had an important effect on enzymic activity and its change with repeated use of the enzyme in both systems, and the porosity could be controlled by the type of solvent and its composition.

The microscopic pictures of the pore structure produced at various different solvent compositions are shown in *Figure 7*. The pore diameter decreased but the number of pores increased when water was replaced by *p*-dioxane, perhaps due to the difference in crystallization properties in relation to the solubilities of monomer and enzyme. However the dependence of the activity on the pore factors and water content in the *p*-dioxane system were not essentially different from those in the water system previously reported[4]. This fact shows that the porosity is the most dominant factor affecting immobilization in these systems and porosity can be attained with any crystallizable solvent. The immiscible solvent can also be utilized for immobilization as a pore inducing component, though there was a greater tendency to enzyme leakage than in the case of miscible solvent system. This result suggested that in principle, immobilization by a hydrophobic monomer–water system would be also possible. It was noticed that the polymer formed at low monomer concentrations in the immiscible solvent system took a microsphere form which was expected to be advantageous for the surface enzyme reaction with the substrate. The microscopic picture of the n-decane system confirmed the increase of smaller pores at a low monomer concentration and also the presence of an independent pore structure

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