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Mechanism of Immobilization of Enzymes by Radiation-Induced Polymerization of Glass-Forming Monomers

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ABSTRACT

Immobilization of enzymes by radiation-induced polymerization has been studied at low temperatures by use of various hydrophilic and hydrophobic glass-forming monomers such as hydroxyalkyl methacrylate and poly(ethylene glycol diacrylate). Activity yield of the immobilized enzyme depends on the monomer concentration in polymerization. In the immobilized enzymes with strongly hydrophobic matrices, the activity shows a maximum at an optimum monomer concentrations in a certain stage of repeated usage for the enzyme reaction. The decrease of activity with repeated use is very little in strongly hydrophobic systems, particularly in diethylene glycol diacrylate polymer matrices. The hydrophobic polymer-enzyme composite has the microsphere form. In the present method, a model scheme for immobilization mechanism is proposed, which is compared with that formed in the polymerization of a nonglass-forming monomer system and also by the solution polymerization at higher temperatures.

INTRODUCTION

The authors have studied the radiation-induced polymerization of glass-forming monomers at low temperatures and its application to the entrapping of biofunctional substances in polymer [1-4]. The proposed method consists of mixing a glass-forming monomer with a solution or suspension of biofunctional substance (such as enzyme and drug) in a crystallizable solvent (such as water or organic solvent) and of polymerizing it by irradiation at low temperatures. The obtained biofunctional polymer composite features porous structure due to the space occupied by crystallized solvent in the supercooled and polymerized matrix at low temperatures. This pore structure favorably influences the activity of the biofunctional composite. For biofunctional polymer materials, the immobilization of enzymes has been studied extensively, and various methods of immobilization have been proposed by other workers [5, 6]. The entrapping method is one of the more promising, because it may be applicable generally to enzymes under relatively mild conditions.

However, hitherto only hydrophilic polymer matrices have been used, since an enzyme is entrapped in a network matrix and the substrate aqueous solution diffuses into it for the enzyme reaction. In the present method reported, the use of less hydrophilic or hydrophobic monomer (polymer) is possible, because much enzyme is entrapped on surface of the pore structure. That is, an enzyme is isolated from the water phase, dispersed on a supercooled monomer phase by ice crystallization, and trapped on the matrix surface by polymerization at low temperatures. Various conventionally hydrophilic and hydrophobic glass-forming monomers (and polymers) were tested as matrices to ascertain their characteristics and applicability for the present method.

MATERIALS AND METHODS

Materials

The polyethylene glycol diacrylate series, $CH_2=CHCOO(CH_2CH_2O)_n$-$OCCH=CH_2$ (n = 2, 4, 9 and 14) and the hydroxyalkyl methacrylate series $CH_2=C(CH_3)COO(CH_2)_nOH$ (n = 2, 4,and 6) as carriers were obtained from the Shin-Nakamura Chemical Co., Ltd. and purified by distillation according to conventional methods. Glucoamylase from Aspergillus niger and 1% maltose solution (0.1 M acetate buffer solution, pH 4.5) were the same as in the accompanying report [7].

Preparation of Immobilized Glucoamylase

The enzyme (0.8 μg) was dissolved in 0.1 M acetate buffer solution (pH 4.5) and then a monomer was added to this enzyme solution with the total volume adjusted to 1 ml. The monomer concentration is given as:

$$\text{Monomer concentration (\%)} = \frac{\text{Monomer (ml)}}{\text{Monomer (ml)} + \text{Buffer (ml)}} \times 100$$

The enzyme-monomer solution was put in an 8-mm diameter glass ampoule and then this ampoule was sealed off under a vacuum of 10^{-3} Torr. Immediately after shaking, the ampoule was frozen at -78° C (Dry Ice-ethanol). The hydrophobic monomer system may be homogeneous by this procedure. The γ-irradiation was carried out at -78°C for 1 hr at a dose rate 5×10^5 rad/hr from a ^{60}Co source. After irradiation, the immobilized enzyme composite obtained was treated as follows. The spongelike gel composite obtained from the hydrophilic monomer system was cut into 8 mm diameter and 2 mm thickness slices. The granular composite obtained from the hydrophobic monomer system was not cut. The hard, glassylike composite obtained at 100% monomer concentration was crushed finely. In any case, the composites obtained were all used for enzyme reactions as-polymerized, without further drying.

Assay of Glucoamylase Activity

The enzyme reaction was carried out by shaking the mixture of 5 ml of 1% maltose solution and the glucoamylase immobilized at 45°C for 30 min in a batch reaction. The glucose formed was determined quantitatively by the same method as described in the accompanying report [7].

Determination of Water Content of the Composite

The water contents of composites polymerized before or after drying were measured by the method described elsewhere [7].

RESULTS AND DISCUSSION

Hydrophilic Properties of Monomers and Polymers

In the monomers expressed by the formula $CH_2{=}CHCOO(CH_2CH_2O)_n$-$OCCH{=}CH_2$ [poly(ethylene glycol diacrylate) series], the hydrophilic

property increases with increase of the number of oxyethylene unit (n). The monomer having oxyethylene unit number n = 9 or higher is completely soluble in water, while those with n = 4 or lower is slightly soluble or insoluble in water. On the other hand, in the monomer series expressed as $CH_2=C(CH_3)COO(CH_2)_nOH$ (hydroxyalkyl methacrylate series), the hydrophilicity decreases with increase of the number of methylene units (n); monomers having n = 4 or lower show complete solubility in water, while those having n = 6 or higher are insoluble in water. These monomers are all glass-forming monomers which form the easily stable supercooled state at low temperatures.

Saturated water contents absorbed in polymers of the two types of monomers are shown as a measure of hydrophilic property in Table 1.

Micrographs of the Polymer-Enzyme Composite

The appearance of the polymer-enzyme composites differs substantially with monomer concentration and also with hydrophilicity or hydrophobicity of the monomer system. The results are summarized in Table 2.

Optical micrographs of the composites from hydrophobic monomer were taken by a microscope Model Nikon F, Nippon Kogaku Co., Ltd., which are given in Fig. 1. The hydrophobic composites are in the microsphere at relatively low monomer concentrations, and obtained as very fine powders by drying. This is quite different from hydrophilic polymers in a porous sponge. Certainly, this microsphere

TABLE 1. Hydrophilicity of Various Glass-Forming Pure Polymers[a]

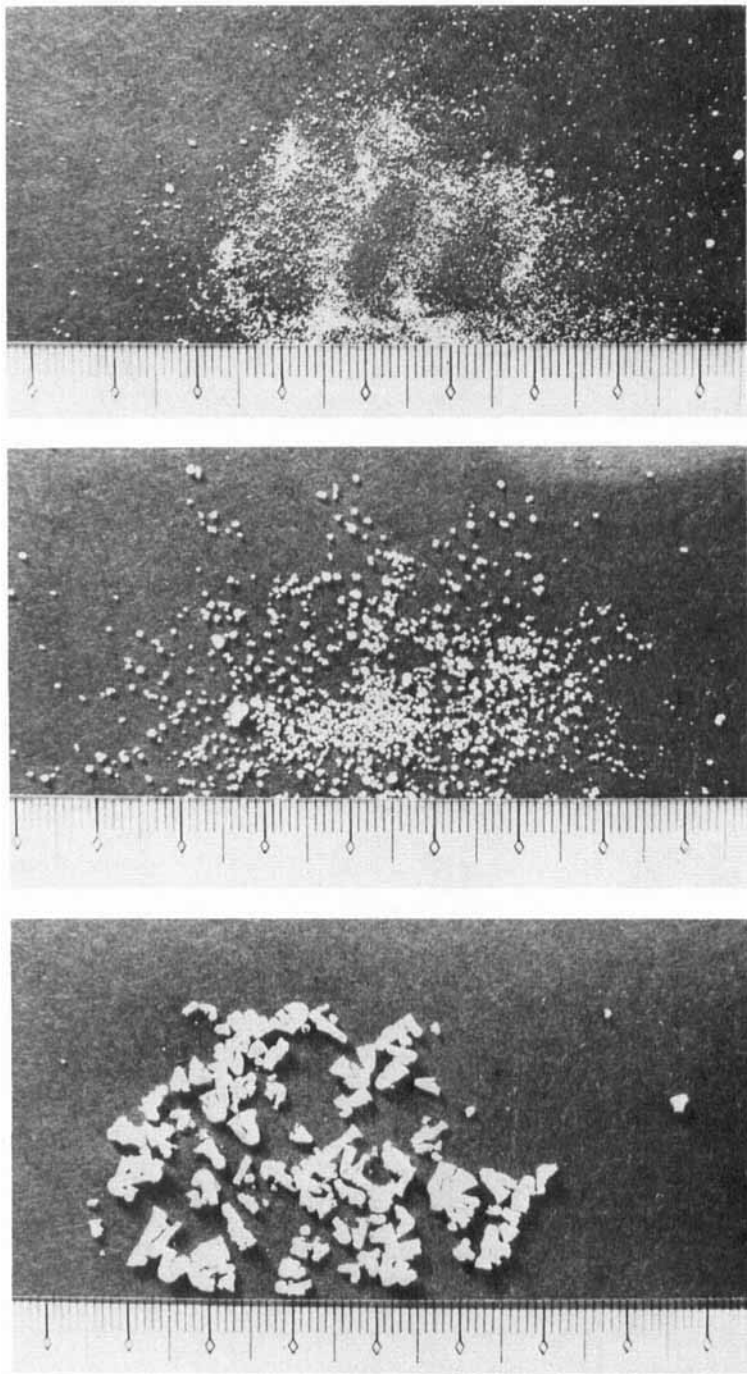
Monomer		
Formula	n	Water content (%)
$CH_2=CHCOO(CH_2CH_2O)_nOCCH=CH_2$	2	2.5
	4	10.0
	9	33.0
	14	45.0
$CH_2=C(CH_3)COO(CH_2)_nOH$	2	26.0
	4	21.5
	6	13.5

[a]The pure polymers (100% monomer concentration) were prepared under the following conditions: 5×10^5 rad at -78°C, in vacuo.

TABLE 2. Appearance of the Polymer-Enzyme Composites Obtained by Radiation-Induced Polymerization at low Temperatures in $CH_2=C(CH_3)COO(CH_2)_nOH$-Water-Enzyme Systems

Monomer concentration (%)	Methylene unit number	
	n = 2[a]	n = 6[b]
5	Soft spongelike matrix (block)	Hard fine granular matrix
10	Soft spongelike matrix (block)	Hard fine granular matrix
15	Soft spongelike matrix (block)	Hard fine granular matrix
20	Soft spongelike matrix (block)	Hard fine granular matrix
25	Soft spongelike matrix (block)	Hard fine granular matrix
30	Soft spongelike matrix (block)	Hard small granular matrix
35	Soft spongelike matrix (block)	Hard small granular matrix
40	Soft spongelike matrix (block)	Hard small granular matrix
45	Soft spongelike matrix (block)	Hard ricelike matrix
50	Hard, spongelike matrix (block)	Hard ricelike matrix
55	Hard, spongelike matrix (block)	Hard ricelike matrix
60	Hard, spongelike matrix (block)	Hard plasterlike matrix (block)
65	Hard, spongelike matrix (block)	Hard plasterlike matrix (block)
70	Hard, spongelike matrix (block)	Hard plasterlike matrix (block)
75	Hard, spongelike matrix (block)	Hard plasterlike matrix (block)
80	Hard, spongelike matrix (block)	Hard plasterlike matrix (block)
85	Hard, spongelike matrix (block)	Hard plasterlike matrix (block)
90	Hard transparent glassy matrix (block)	Hard transparent glassy matrix (block)
95	Hard transparent glassy matrix (block)	Hard transparent glassy matrix (block)
100	Hard transparent glassy matrix (block)	Hard transparent glassy matrix (block)

[a]Hydrophilic monomer.
[b]Hydrophobic monomer.



(a)

(b)

(c)

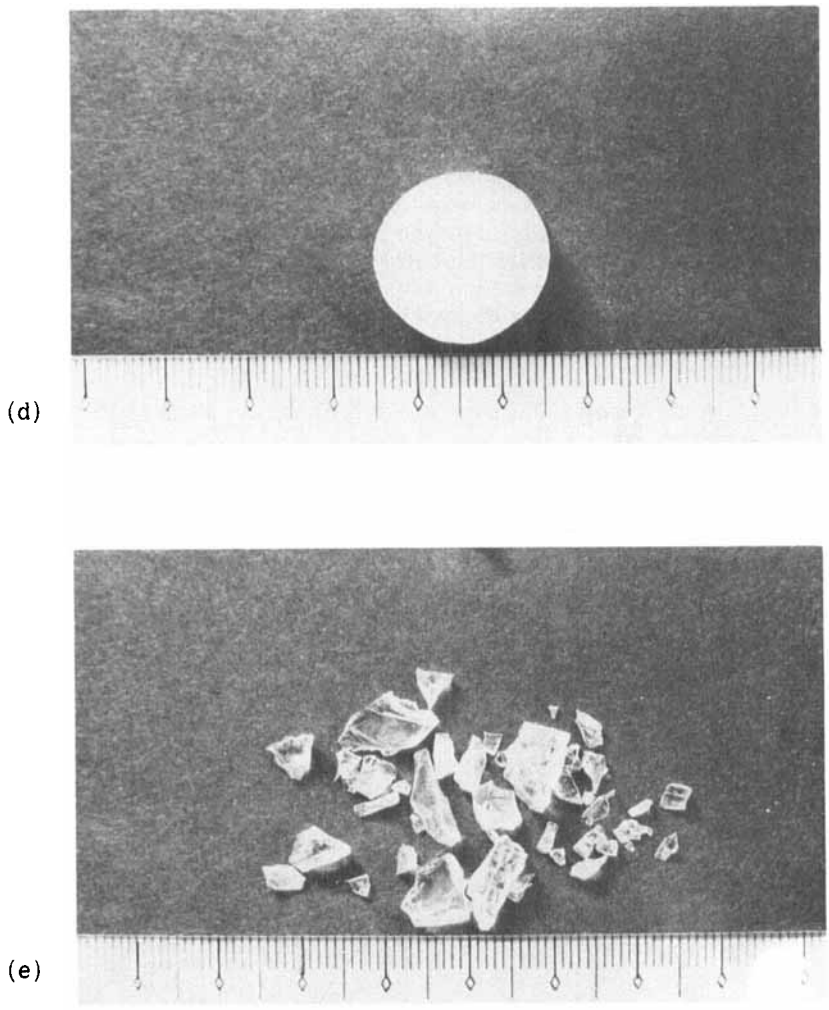


FIG. 1. Photographs of polymer formed in hydrophobic system in the dried state at various monomer [$CH_2=C(CH_3)COO(CH_2)_6OH$] concentrations: (a) 10%; (b) 30%; (c) 50%; (d) 70% (circular block matrix); (e) 100% (crushed matrix). The monomer solution was charged into a 20-mm diameter glass ampoule, and the ampoule was then irradiated for 1 hr at a dose rate of 5×10^5 rad/hr at -78°C, in vacuo.

structure is due to the structure of the suspension of the monomer-in-water type in polymerization phase. It is probable that the enzyme is trapped on the surface of the microsphere, because possibly most of the enzyme is isolated and moves to the monomer-water interface or monomer surface from the water phase on recrystallization from water. Moreover, these hydrophobic microspheres showed considerable activity without absorbing water and swelling, so the enzyme should exist on the matrix surface. This microsphere structure is advantageous for the surface reaction to proceed rapidly without the need for substrate diffusion into the polymer matrix. Such surface trapping especially benefits the heterogeneous reaction with a water-insoluble high molecular weight substrate. Furthermore, it is not necessary to pulverize the composite to increase the effective surface area for reaction.

Relation between Enzymatic Activity and Monomer Concentration

The relation between enzymatic activity of the polymerized composite and monomer concentration in the polymerization is shown for various hydrophilic and hydrophobic systems in Figs. 2 and 3. According to the results of Fig. 2, the activity at first is relatively small, and it changes slightly with repeated use (the difference in activity between the first reaction and multiply repeated reaction is small or negligible) in less hydrophilic (n = 4 in Fig. 2) and

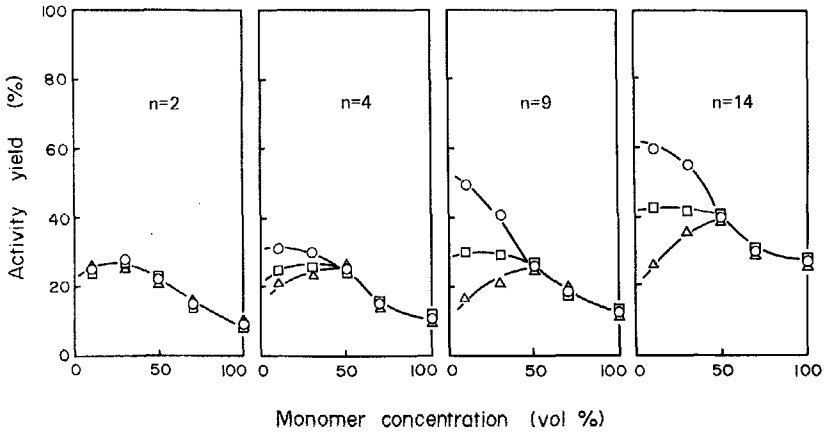


FIG. 2. Relationship between the monomer concentration and the activity yield of immobilized glucozmylase in the poly(ethylene glycol diacrylate) series for various numbers of batch reactions: (○) 1; (□) 5; (△) 15.

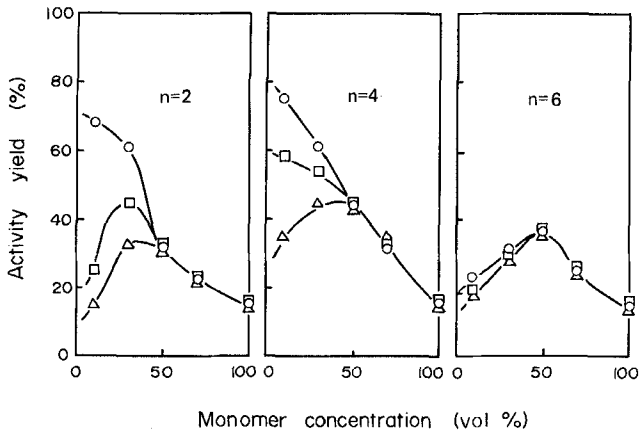


FIG. 3. Relationship between the monomer concentration and the activity yield of immobilized glucoamylase in the hydroxyalkyl methacrylate series for various numbers of batch reactions: (○) 1; (□) 5; (△) 15.

hydrophobic (n = 2 in Fig. 2; n = 6 in Fig. 3) composites. This tendency is observed in all composites obtained over the whole monomer concentration range (even at very low monomer concentration). On the other hand, in relatively strongly hydrophilic composites, the activity yield shows no or small change with repeated use only in the composites obtained at high monomer concentrations. In the hydrophilic composite obtained at low monomer concentration, the initially high activity decreases rapidly with repeated use, due to enzyme leakage from the matrix having an excessively porous structure.

On the other hand, in hydrophobic composites such as the diethylene glycol diacrylate system, no leakage of enzyme was observed in the matrices obtained at very low monomer concentrations. It is thus suggested that the trapped yield of enzyme is smaller but the enzyme leakage is suppressed in the hydrophobic composites, compared with the case of hydrophilic composites, possibly due to the lack of pore structure and the lower swelling of matrix in the former. A similar tendency is observed in the hydroxyalkyl methacrylate series, as shown in Fig. 3. Even in hydrophilic systems, enzyme leakage is hardly observed at monomer concentrations higher than about 50%. The maximum activity is attained at a certain monomer concentration in the hydrophilic systems. This may be due to the balance of two factors affecting activity: the composite of low monomer concentration is very porous and the effective surface area in contact and reacting with the substrate is large, while the enzyme leakage through pores of the matrix is also large. On the other hand, in composites obtained at relatively high monomer concentrations, the effective

surface area is smaller (the occluded enzyme in the matrix could not react with the substrate); thus enzyme leakage is suppressed. These two factors, enzyme leakage and effective surface area, both being functions of the monomer concentration, act in opposite directions for activity. Perhaps the best balance is attained at a certain porosity of the composite under certain monomer concentrations.

On the other hand, the maximum activity in hydrophobic system may be explained as follows. In the hydrophilic monomer system, the enzyme is dissolved uniformly in the aqueous solution of monomer, and it is then partly isolated at the monomer-ice intersurface and partly remains in the monomeric matrix, by ice crystallization at low temperatures. A large part of the ice remains in the pores of the spongelike matrix after polymerization. There is thus a small loss of enzyme during immobilization, but a certain leakage occurs with use from the matrix-pore interface (leakage of the freely existed enzyme in interface). On the other hand, in the hydrophobic monomeric system (suspension of water-monomer), the enzyme is all dissolved in the water phase (not in the monomer phase) and precipitates at the monomer-ice interface on cooling. In this case, a large part of the ice (water) is removed from microspheres of the composite after polymerization, and considerable part of enzyme in the interface is removed and lost with water before use. The loss of enzyme is larger in the microspheres obtained at low monomer concentrations, due to the smaller polymer content. This may be the reason for the decrease of activity with a decrease of the monomer concentration. On the other hand, the suspension structure of the monomeric system changes from monomer-in-water type to water-in-monomer type in the region of relatively high monomer concentration. Moreover, the ratio of independent closed cells in the matrix occupied by water particles increases, and the diffusion of substrate into the matrix through the open continuous cells is difficult. This may be the reason for the decrease of activity with increase of the monomer concentration. As a result, the monomer concentration dependence of the activity has an apparent maximum.

Model Scheme for Immobilization Mechanism

The proposed model schemes for the immobilization mechanism are shown in Figs. 4-7. Figure 4 is a model scheme for low-temperature immobilization by hydrophilic glass-forming monomer and Fig. 5 is the model scheme for low temperature immobilization by hydrophobic glass-forming monomer. In the former, some part of the enzyme remains in the freely isolated state in pores or at the pore-matrix interface to leak out easily in repeated use, while a considerable part of the enzyme is firmly trapped on the surface of matrix and some part of the enzyme is buried inside matrix without contributing the activity. In the latter, some part of the enzyme is freely isolated to be lost easily out of polymer spheres or continuous matrix,

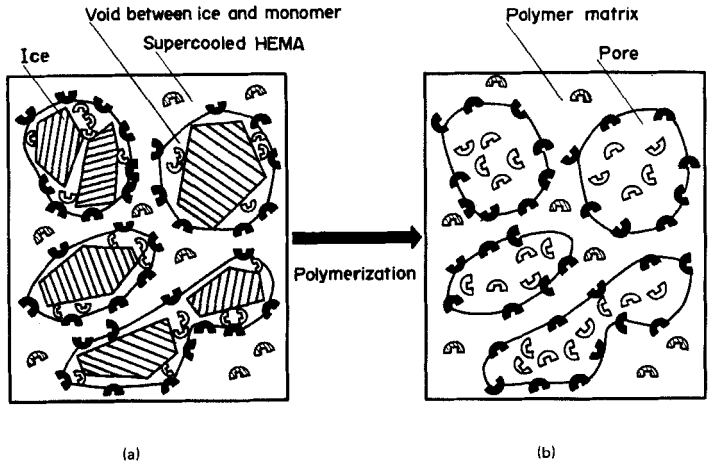


FIG. 4. Model schemes for immobilization by hydrophilic glass-forming monomer system at low temperatures for (a) a cooled monomer system and (b) a polymerized system: (ᗢ) entrapped enzyme; (ᗢ) entrapped enzyme inside matrix; (ᗢ) freely isolated enzyme.

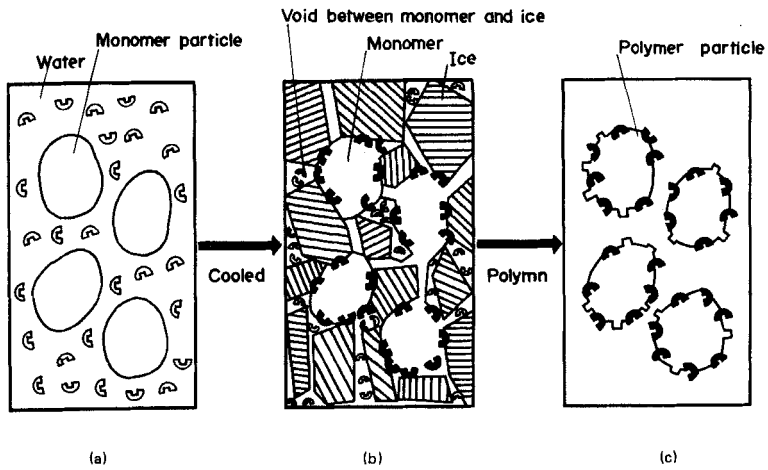


FIG. 5. Model scheme for immobilization by hydrophobic glass-forming monomer system at low temperatures: (a) noncooled monomer system; (b) cooled monomer system; (c) polymerized system.

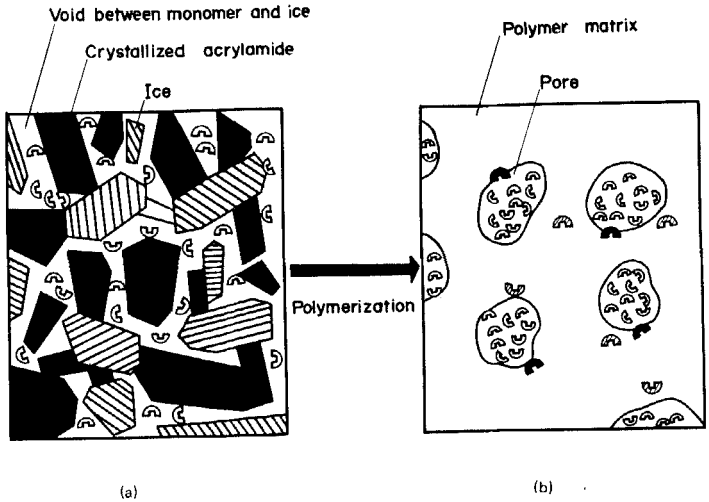


FIG. 6. Model scheme for immobilization by hydrophilic nonglass-forming monomer system at low temperatures: (a) cooled monomer system; (b) polymerized system.

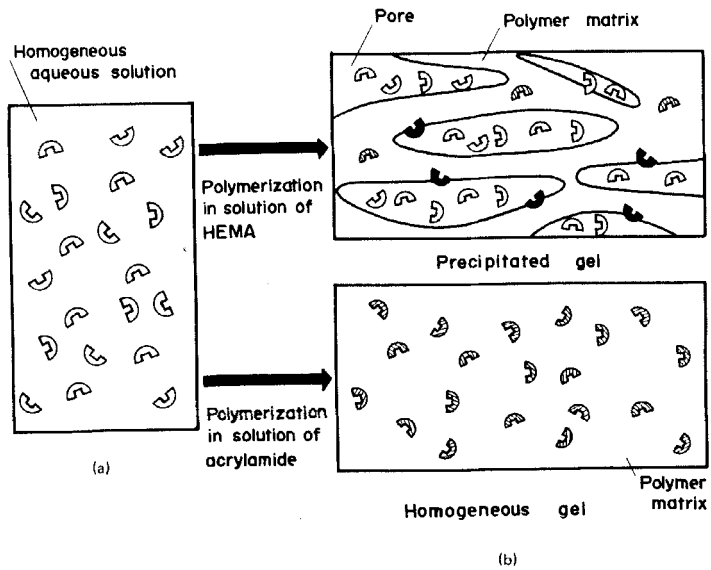


FIG. 7. Model scheme for immobilization by hydrophilic glass-forming monomer, $CH_2=C(CH_3)COO(CH_2)_2OH$ (HEMA) and hydrophilic nonglass-forming monomer, $CH_2=CHCONH_2$ (acrylamide) systems at elevated temperatures: (a) noncooled monomer system; (b) polymerized system.

and other considerable part of the enzyme is trapped on the surface of microspheres or matrix. The free enzyme outside the polymer is lost with water in the polymer isolation step, while the remaining enzyme bound on to polymer surface does not leak.

For comparison, Fig. 6 shows the model scheme for immobilization at low temperatures by a nonglass-forming monomer such as acrylamide. In this case, the freely isolated enzyme increases due to the crystallization of both monomer and water, and enzyme is entrapped loosely and unstably with post-polymerization of the crystalline monomer and with swelling of the formed polymer, so that there occurs substantial enzyme leakage with repeated use.

Figure 7 shows model schemes for two types of immobilization on solution polymerization at relatively high temperature, such as room temperature. One is heterogeneous aqueous solution polymerization, such as in the case of 2-hydroxyethyl methacrylate, in which the formed polymer precipitates from the monomeric phase due to less hydrophilic property of the polymer. The other is homogeneous solution polymerization, such as that of acrylamide giving strong hydrophilic polymer. In the former case, a large part of the enzyme remains and is concentrated in the water phase with precipitation of the polymer, so that only a small part is trapped rather unstably in the polymer phase. As a result, the activity yield is low, and the enzyme leakage is large in this case. In the latter, all the enzyme is completely entrapped in the swollen homogeneous polymer gel with water. In this case, the enzyme reaction is completely controlled by diffusion of the substrate into the matrix, and the reaction rate is very slow. If the diffusivity in the matrix increases to heighten the reaction rate with increasing hydrophilic property (swelling property), enzyme leakage becomes very active and the activity retention decreases rapidly with repeated use.

Consequently, it is characteristic and an advantage of the present method that considerable part of the enzyme is trapped on surface of the polymer matrix having a porous spongelike or microsphere structure formed by suspension structure of the monomeric phase, which consists of ice and supercooled monomer at low temperatures. A glass-forming (supercooling) property of the monomers is necessary, not only to obtain high polymerizability but also to give effective pore or sphere structure at low temperatures.

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