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# Immobilization of Enzymes by Radiation-Induced Copolymerization of 2-Hydroxyethyl Methacrylate and Other Hydrophilic or Hydrophobic Comonomers

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## ABSTRACT

Immobilization of enzymes by radiation-induced copolymerization was studied at low temperatures by use of various comonomer systems consisting of 2-hydroxyethyl methacrylate and other more hydrophilic or hydrophobic comonomers. The matrices obtained by copolymerization with more hydrophilic and more hydrophobic comonomers decreased the porosity in the matrix equally. However, the activity yield of the immobilized enzyme showed different changes with repeated use in the more hydrophilic and more hydrophobic matrices. That is, the initial activity decreased rapidly with repeated use owing to the enzyme leakage from the matrix, in the increased hydrophilic matrices. On the other hand, in the more hydrophobic matrices enzyme leakage was completely retarded and activity did not change with repeated use. Moreover, the activity yield showed a maximum at a certain monomer composition in the copolymerization with hydrophobic comonomer. Finally, it was found that the maximum activity yield of the hydrophobic matrices was larger in general than that of the hydrophilic copolymer matrices.

## INTRODUCTION

The radiation-induced polymerization at low temperatures of glass-forming monomers which have a stable supercooling property and a large polymerizability at low temperatures has been studied previously [1, 2]. The application of this polymerization to the trapping of biofunctional substances by polymerizing a mixture of glass-forming monomer such as 2-hydroxyethyl methacrylate, biofunctional materials such as enzymes, microbial cells, and drugs, and crystallizable solvents (usually water) at low temperatures was investigated [3-5]. The use of various hydrophilic and hydrophobic glass-forming monomers as carrier matrices was tried.

A porous or spherical structure was characteristic of the matrix obtained by the present method owing to the suspension structure consisting of ice and a supercooled monomer at low temperatures. These structures affected the biofunctionality of the polymer composite markedly. It was also characteristic of this method that a considerable part of the biofunctional substance could be trapped mainly on the surface region of the porous or spherical structure in the matrix. This is advantageous for the biochemical reaction on a polymer surface, because diffusion of the substrate into the polymer matrix gel is not so necessary for the biochemical reaction, in contrast to the reaction by hydrogel type matrix used in the conventional entrapping immobilization method in which the enzymes were entrapped inside the cross-linked network of the matrix.

In the present method, we can choose a suitable carrier from a wide range of hydrophilic or hydrophobic vinyl monomers. Furthermore, a suitable combination of those monomers is also utilizable. The change in the hydrophilicity of the matrix by copolymerization might be effective for control of the porous structure and activity. In this report, the control of the hydrophilic and hydrophobic property in the matrix was investigated in relation to the porous structure and enzymatic activity of the matrix by means of copolymerization of 2-hydroxyethyl methacrylate with other comonomers.

## MATERIALS AND METHODS

### Materials

The glucoamylase from Aspergillus niger as an enzyme, 1% maltose solution (pH 4.5) as a substrate, and 2-hydroxyethyl methacrylate (HEMA) as a monomer used in this work were the same as described in the previous report [3]. Hydrophilic monomers such as hydroxyethyl acrylate (HEA), acrylamide (AAm), and N-vinyl-2-pyrrolidone (NVP) were obtained from the Tokyo Kasei Kogyo Co., Ltd. and purified by distillation or recrystallization. Hydrophobic monomers such as hexanediol monomethacrylate

(HDMM), diethylene glycol dimethacrylate (DGDA), and methyl methacrylate (MMA) were obtained from the Shin-Nakamura Chemical Co., Ltd. and purified by distillation.

## Preparation of Immobilized Glucoamylase

The enzyme (0.8 $\mu$g) was dissolved in 0.5 ml of 0.1 M acetate buffer solution (pH 4.5). Various comonomer compositions (0.5 ml) of HEMA-HEA, HEMA-AAm, HEMA-NVP, HEMA-HDMM, HEMA-DGDA, and HEMA-MMA were added to the above enzyme solution. The monomer concentration was prepared to be 50% of the total mixture (1 ml in volume). The enzyme-comonomer solution was charged into an 8 mm diameter glass ampoule. The ampoule was sealed off under a vacuum of $10^{-3}$ Torr and shaken enough to form a suspension (hydrophobic system) or a homogeneous solution (hydrophilic system). Then, the sealed ampoule was immersed in a Dewar flask kept at -78°C by Dry Ice-methanol and irradiated by $\gamma$-rays at -78°C for 1 hr at a dose rate of $5 \times 10^5$ rad/hr from a $^{60}$Co source.

After irradiation, the immobilized enzyme composite obtained was cut to 8 mm diameter, 2 mm thick slices in the case of hydrophilic composition. The granular composite obtained from the hydrophobic composition was used without cutting. In all cases, the composites were used for the enzyme reaction in the as-polymerized state without drying.

## Assay of Glucoamylase Activity

The batch reaction was carried out by shaking a mixture of 5 ml of 1% maltose solution (pH 4.5) and immobilized glucoamylase at 45°C for 30 min. The glucose formed was determined by measuring the absorption at 505 nm with a Shimazu QV-50 spectrophotometer by using GOD-PODLK obtained from the Nagase Sangyo Co. Ltd., which consists of glucose oxidase, glucose peroxidase and chromogen (coloring matter) and carrying out the following reaction [7]:

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

$$H_2O_2 + \text{dye (Red.)} \xrightarrow{\text{glucose peroxidase}} H_2O + \text{dye (Oxid.)}$$

## Determination of Porous Structure in Polymer Composite

The polymer composite was cut into slices 15-25 $\mu$m thick. The pore structure in polymer composite as polymerized state was observed by optical microscope. Characteristics of the pore structure,

such as average pore diameter, pore number per unit area of polymer composite, and porosity were determined by studying the photomicrographs.

The average pore diameter of a pore in the pore structure was determined from Eq. (1):

$$\text{Average pore diameter } (\mu\text{m}) = 2\left[\frac{\text{Average area of pore}}{\pi}\right]^{1/2} \quad (1)$$

The porosity was defined by Eq. (2):

$$\text{Porosity } (\%) = \frac{\text{Total area of pores}}{\text{Total area of visual field in microscopy (pores and polymer matrix)}} \times 100 \quad (2)$$

Water content in the polymer composite was calculated according to Eq. (3):

$$\text{Water content } (\%) = [(W_s - W_p)/W_s]100 \quad (3)$$

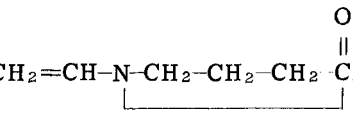
where $W_s$ is the weight of the polymer composite before drying, consisting of weight of matrix and water contained in the matrix and the pore structure; $W_p$ is the weight of the polymer composite after the drying treatment and equals to the weight of matrix itself.

## RESULTS AND DISCUSSION

### Hydrophilic Properties of Polymers Used as Components

Hydrophilic properties of the polymer used as components in this work are shown in Table 1. The hydrophilic property of the copolymer increases on copolymerization of HEMA with HEA, AAm, and NVP, while it decreases on copolymerization with HDMM, DGDA, and MMA. Of these, HEA, HDMM, and DGDA are glass-forming monomers. The other comonomers have no glass-forming property but they can be used with HEMA in a certain composition range without destroying the glass-forming properties.

TABLE 1. Apparent Water Content of Pure Polymers

| | Monomer | Water content (%) |
|---|---|---|
| HEMA | $CH_2=C(CH_3)COO(CH_2)_2OH$ | 26.0 |
| NVP |  | 93.7 |
| AAm | $CH_2=CHCONH_2$ | 84.8 |
| HEA | $CH_2=CHCOO(CH_2)_2OH$ | 45.9 |
| HDMM | $CH_2=C(CH_3)COO(CH_2)_6OH$ | 13.5 |
| DGDA | $CH_2=C(CH_3)COO(CH_2CH_2O)_2OC(CH_3)C=CH_2$ | 2.5 |
| MMA | $CH_2=C(CH_3)COOCH_3$ | 2.1 |

## Effect of Copolymerization of HEMA with More Hydrophilic Comonomers on Porosity and Enzymatic Activity

The various factors related to the properties of the porous structure were estimated by microscopic observation. The effect of the monomer composition of the HEMA-hydrophilic comonomer systems on these pore factors is shown in Figs. 1 and 2. According to these results, the porosity generally tended to decrease with increasing content of strongly hydrophilic comonomer, that is, with increasing hydrophilic property and water content in the copolymer. This result is perhaps attributed to expansion of the polymer matrix by water swelling so as to narrow the pore diameter and to join the individual pores. The pore factors (average pore diameter and pore number) and porosity scarcely decreased in the copolymer with HEA. This may be due to the relatively similar hydrophilic properties of HEMA and HEA.

The changes of the enzymatic activity with repeated use for the enzyme reaction are shown as a function of monomer composition in Figs. 3 and 4. According to the result in Fig. 3, the decrease in activity due to repeated use became quite marked with increasing content of the hydrophilic comonomer. That is, the initial activity yield decreased quickly with repeated use in those strongly hydrophilic systems. On the other hand, in relatively less hydrophilic

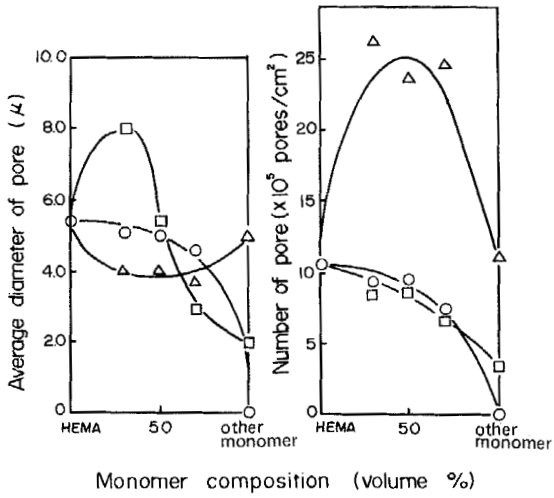



FIG. 1. Effects of monomer composition on pore factors such as average pore diameter and pore number in various HEMA-hydrophilic comonomer systems: ( ○ ) HEMA-NVP monomer system; ( □ ) HEMA-AAm system; ( △ ) HEMA-HEA system. Monomer concentration, 50% monomer-50% acetate buffer solution (pH 4.5).

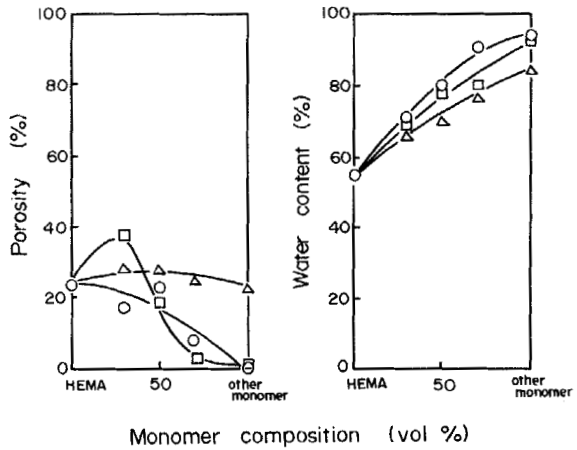



FIG. 2. Effects of monomer composition on porosity and water content in various HEMA-hydrophilic comonomer systems. Experimental conditions same as in Fig. 1.

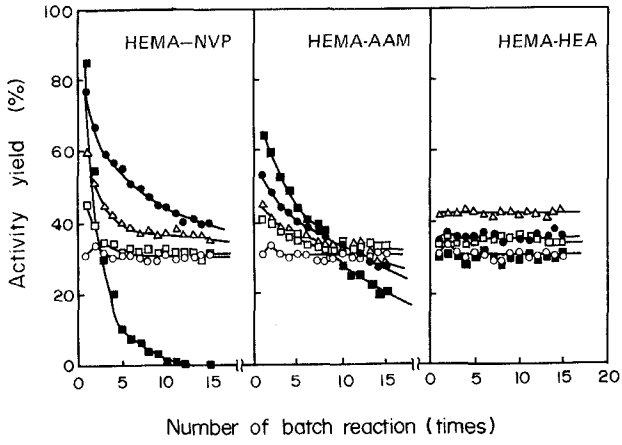



FIG. 3. Effects of number of reaction batches on activity yield of glucoamylase immobilized as a monomer composition in various HEMA-hydrophilic comonomer systems: ( ○ ) 100% HEMA; ( □ ) 70% HEMA-30% comonomer; ( △ ) 50% HEMA-50% comonomer; ( ● ) 30% HEMA-70% comonomer; ( ■ ) 100% other monomer. Monomer concentration, 50% monomer-50% acetate buffer solution (pH 4.5).



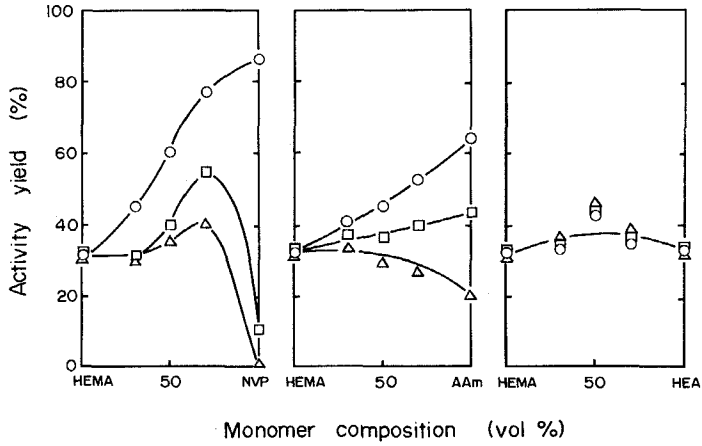



FIG. 4. Effects of monomer composition on activity yield of immobilized glucoamylase in various HEMA-hydrophilic comonomer systems: ( ○ ) 1 reaction batch; ( □ ) 5 reaction batches; ( △ ) 15 reaction batches. Monomer concentration, 50% monomer-50% acetate buffer solution (pH 4.5).

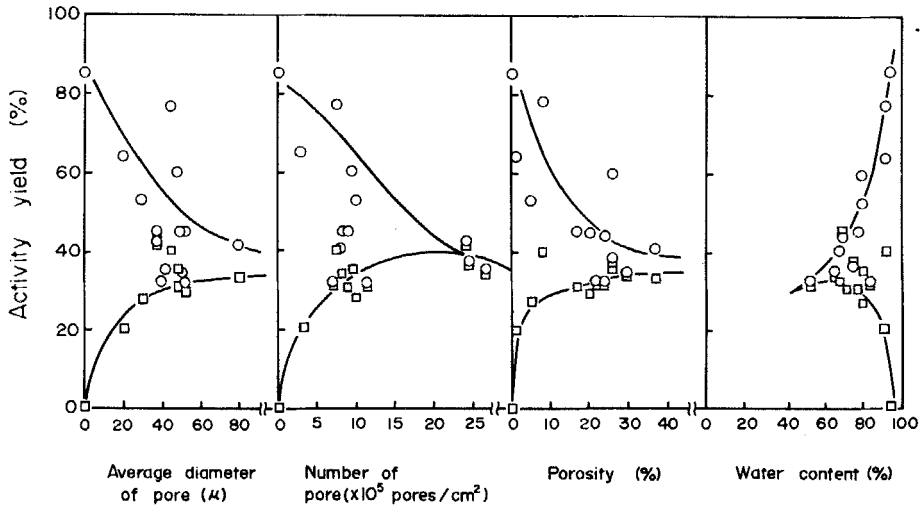



FIG. 5. Effects of pore factors on activity yield of immobilized glucoamylase plotted by using the data given in Figs. 1-4, in various HEMA-hydrophilic comonomer systems: ( ○ ) 1 reaction batch; ( □ ) 15 reaction batches.

systems, such as the HEMA-HEA system, no decrease of the initial activity was observed at all in the composition range studied as long as the monomer concentration relative to water was kept at a relatively high range such as 50% in the studied systems. The same result is shown in Fig. 4 as a function of the monomer composition. The difference in activity between the first reaction and the later reactions indicates a decrease of activity with repeated use. This difference markedly increased in strongly hydrophilic compositions. It was ascertained by analysis of the enzyme content in the solution after the reaction that the decrease of the initial activity with repeated use could be attributed to leakage of the enzyme isolated freely in the pore structure. In the case of strongly hydrophilic matrices, it is probable that enzyme leakage is promoted by strong swelling of the polymer, not only from the pore space but also from near the surface area of the matrix. This is probably the reason for the results described above.

The relations between the enzymatic activity and the pore factors in HEMA-hydrophilic comonomer systems are shown in Fig. 5. The enzyme leakage can be evaluated by the difference in activity between the first and the 15th repeated use. This difference increased with decreasing pore diameter, pore number, and porosity in the strongly hydrophilic systems. On the other hand, in relatively less hydrophilic systems such as the pure HEMA system and HEMA-HEA system,

enzyme leakage decreased with decreasing values of those pore factors. These facts suggest that in strongly hydrophilic systems, enzyme leakage occurs not only from the pore but also from inside the matrix (perhaps from near the surface area of the porous matrix) by swelling of the polymer. In contrast, leakage of the enzyme takes place mainly by diffusing out of freely isolated enzyme in the pore space in less hydrophilic matrix systems. This is probably the reason for the different results in dependence of activity retention with repeated use on the pore factors or porosity.

## Effect of Copolymerization of HEMA with More Hydrophobic Comonomers on Porosity and Enzymatic Activity

The relation between the pore factors and the monomer composition in HEMA-hydrophobic comonomer systems is shown in Figs. 6 and 7. The complicated dependence of monomer composition on pore number in Fig. 6 can be reduced to phase changes of the monomeric and polymeric systems with monomer composition. That is, the monomeric phase of these systems gradually changes to monomer in water or water in monomer suspension from the homogeneous solution with increasing hydrophobic monomer compositions. Therefore, the formed polymer changes from a porous, spongelike polymer to a

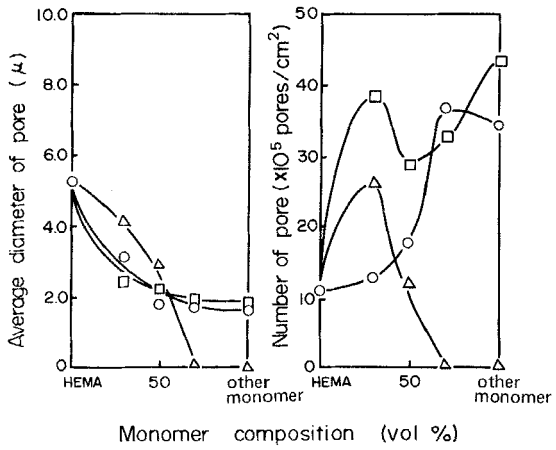



FIG. 6. Effects of monomer composition on pore factors such as average pore diameter and pore number in various HEMA-hydrophobic comonomer systems: ( ○ ) HEMA-HDMM; ( □ ) HEMA-DGDA; ( △ ) HEMA-MMA. Monomer concentration, 50% monomer-50% acetate buffer solution (pH 4.5).

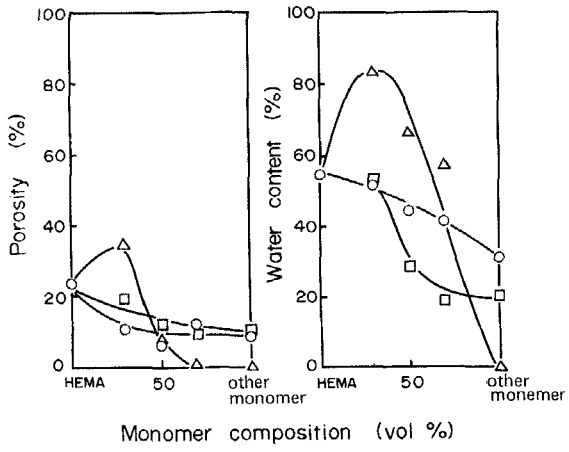



FIG. 7. Effects of monomer composition on porosity and water content in various HEMA-hydrophobic comonomer systems. Experimental conditions same as in Fig. 6.



microsphere particle, corresponding to the change of monomeric phase at hydrophobic compositions. However, the formed hydrophobic polymer in the as-polymerized state has some apparent water content as shown in Fig. 7. Because, in this monomer concentration of 50% water, the monomeric system does not form a complete monomer-in-water suspension but contains some water-in-monomer structure. Then, the formed polymer is not a completely independent microsphere, but has some continuously jointed matrix structure including water, though the microsphere particle is easily obtained by drying this polymer. The perfect microsphere polymer in its polymerized state is obtained at a lower monomer concentration, at which the monomeric systems form perfect monomer-in-water suspensions. Generally, the copolymer system with hydrophobic comonomers showed a tendency for decreased pore diameter and porosity as the content of the hydrophobic comonomer increased. The reason may be attributable to the change of the monomeric phase from solution to suspension and also a change in the polymeric phase from spongelike gel to sphere particle. In the HEMA-MMA system, the polymerizability decreased with increasing MMA content and was lost at a certain MMA composition owing to the crystallization of the MMA.

The change of enzymatic activity with repeated use at various monomer compositions is shown in Fig. 8. The activity decrease due to enzyme leakage with repeated use was hardly observed in each system at all monomer compositions, because freely isolated enzymes scarcely exist in the pore in this monomer concentration

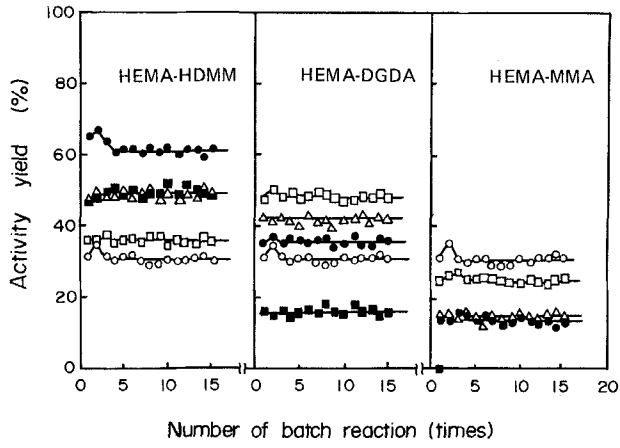



FIG. 8. Effects of number of reaction batches on activity yield of glucoamylase immobilized as a function of monomer composition in various HEMA-hydrophobic comonomer systems: ( ○ ) 100% HEMA; ( □ ) 70% HEMA-30% comonomer; ( △ ) 50% HEMA-50% comonomer; ( ● ) 30% HEMA-70% comonomer; ( ■ ) 100% other monomer. Monomer concentration, 50% monomer-50% acetate buffer solution (pH 4.5).



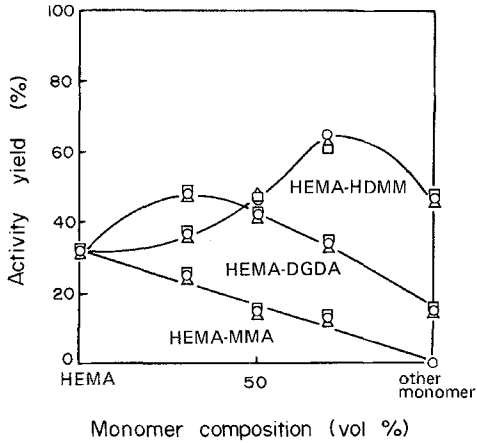



FIG. 9. Effect of monomer composition on activity yield of immobilized glucoamylase in various HEMA-hydrophilic comonomer systems: ( ○ ) 1 reaction batch; ( □ ) 5 reaction batches; ( △ ) 15 reaction batches.

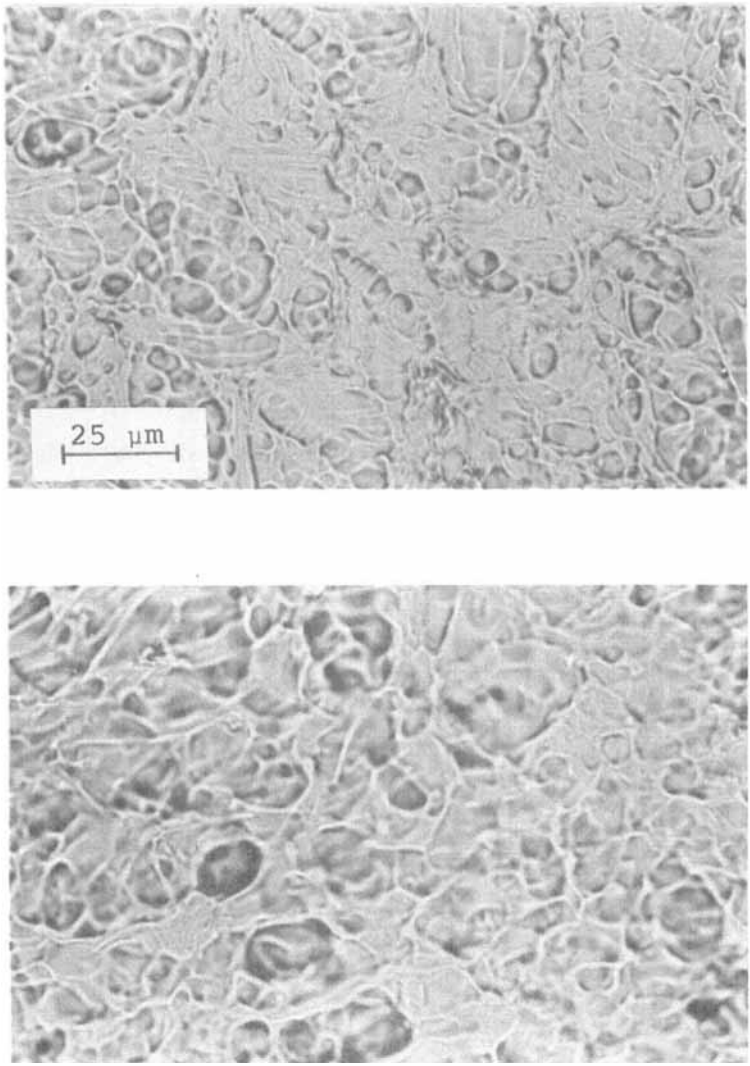
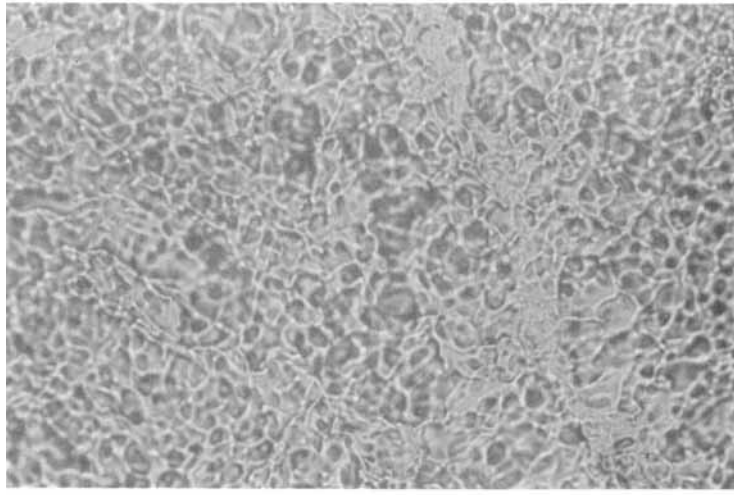
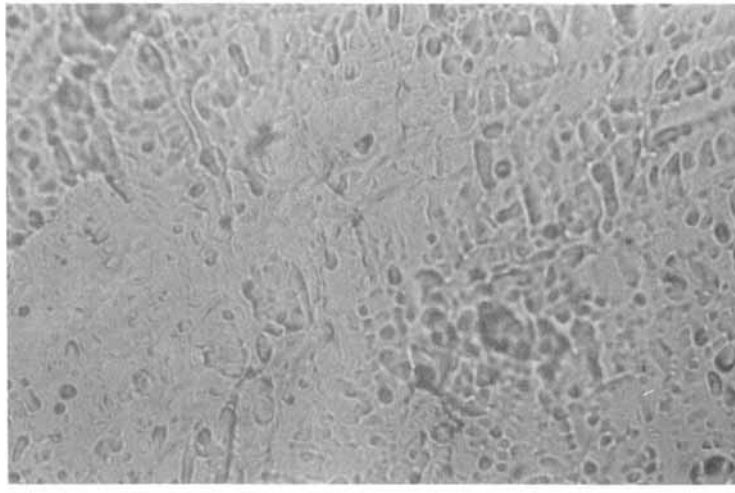



FIG. 10. Optical photomicrographs of pore structure in polymer matrix as polymerized state obtained by copolymerization of HEMA with other monomers: (a) 70% HEMA-30% NVP; (b) 70% HEMA-30% AAm; (c) 70% HEMA-30% DGDA; (d) 70% HEMA-30% HDMM; (e) 30% HEMA-70% AAm; (f) 30% HEMA-70% DGDA.



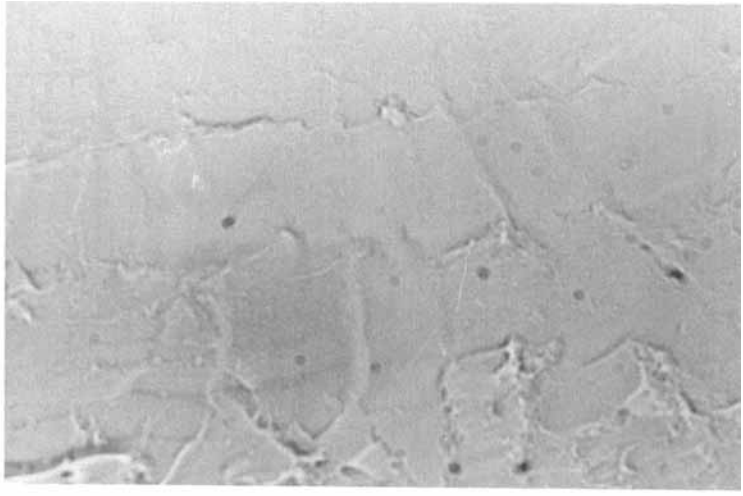
(c)



(d)

FIG. 10. (continued)

(e)



(f)

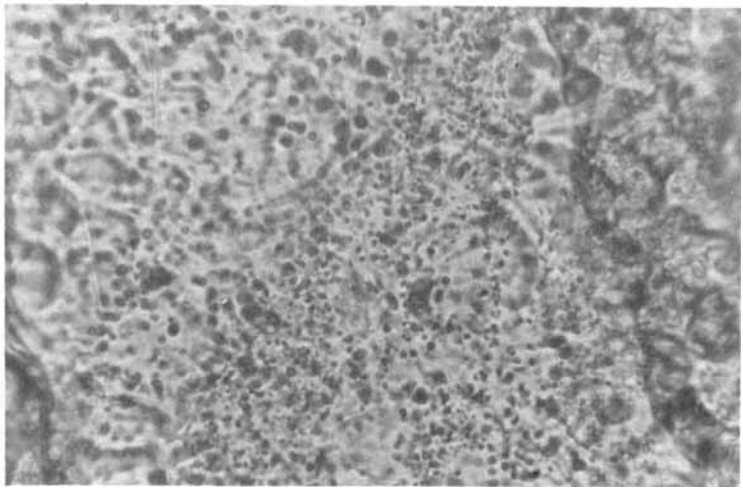


FIG. 10. (continued)

(50%), even in the pure HEMA system, and much more in the hydrophobic copolymer system which is less porous than the pure HEMA system.

The activity yield of the hydrophobic copolymer systems was larger than that of the hydrophilic systems in general and showed a maximum at a certain hydrophobic monomer composition in HEMA-HDMM and HEMA-DGDA systems, as shown in Fig. 9. These results may be due to some hydrophobic affinity between the hydrophobic bond in enzymes and that in monomer or polymer, because the contribution of hydrophobic bonding to the activity of the enzyme is known, though the mechanism is not clear. The considerable activity of the hydrophobic systems supports the conclusion that the enzyme is trapped on the surface area of the matrix pore or of the microsphere and that the reaction is carried out on these surface parts without much inner diffusion of substrate into the matrix as in hydrogel-type crosslinked polymers in the conventional entrapping method.

Micrographs of various polymer matrices are shown in Fig. 10. The matrices in Figs. 10a-d show spongelike pore structures, because the monomeric systems are still hydrophilic and hardly form monomer-in-water suspension at those HEMA-rich monomer compositions. The micrographs shown in Figs. 10e and 10f are of the matrices in HEMA-poor monomer compositions. The HEMA-AAm system contains few pores, while the HEMA-DGDA system has many pore structures. This may very well be the reason for the difference in hydrophilicity of the copolymer, that is, swelling by water.



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