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IMMOBILIZATION OF ENZYMES BY RADIATION-INDUCED POLYMERIZATION OF GLASS-FORMING MONOMERS. DOUBLE ENTRAPPING OF ENZYMES IN THE PRESENCE OF VARIOUS ADDITIVES

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Immobilization of enzymes by a double entrapping method was investigated. α-Amylase was trapped by radiation-induced low temperature polymerization of 2-hydroxyethyl methacrylate after being absorbed by various inorganic additives. Drierite showed the most remarkable effect by increased enzymatic activity and decreased activity loss in batch enzyme reactions. Double entrapping of cellulase was tried by preparing an entangled mixture of enzyme and various natural polymers as additives and then polymerizing with the monomeric system at low temperatures. The composite, including enzyme–additive mixture prepared by coprecipitating and coevaporating methods, showed a considerable enhancing effect on enzymatic activity and durability in batch enzyme reactions.

INTRODUCTION

The authors have studied the immobilization of enzymes and microbial cells by radiation-induced polymerization at low temperature (1). Immobilization at low temperature could be carried out conveniently and effectively using hydrophilic and hydrophobic glass-forming monomers, which can be polymerized at low temperatures (2). Moreover, it was found that the polymer obtained by low temperature polymerization was characterized by a large surface area due to the porous structure or microsphere structure. It was the most important characteristic of our method that a considerable portion of enzyme was entrapped on the surface of the polymer matrix. This characteristic was advantageous for the enzyme reaction with substrate, because diffusion resistance of the matrix did not have a serious effect on the reaction rate as observed in hydrogel-type polymers obtained by previous entrapping methods. However, the

considerable enzyme losses observed without entrapping were inevitable concomitants of immobilization in the earlier methods, and so the final product was not satisfactory. Further, enzyme leakage in batch enzyme reactions was observed with polymers obtained using low monomer concentrations due to the excess large porosity of polymer. In this study, the double entrapping method was tried in order to prevent the enzyme loss and its leakage; that is, the entrapping by polymerization of monomer after absorbing on an inorganic absorbent by flocking with a natural high polymer of enzyme was investigated.

MATERIALS AND METHODS

Materials

α-Amylase (*Bacillus subtilis*) and cellulase (*Trichoderma viride*) were obtained from Nagase Sangyo and Kinki Yakult Manufacturing Company, Ltd. Soluble starch, sodium carboxymethyl cellulose (CMC), and 2-hydroxyethyl methacrylate (HEMA) were obtained from Katayama Chemical, Kanto Chemical, and Mitsubishi Gas Chemical Company, Ltd., respectively. The absorbents of silica gel (Nishio Industry Company Ltd.), molecular sieves 4A (Nishio Industry Company, Ltd.), Drierite (W. A. Hamond Drierite Company, Ltd.), agar (Kishida Chemical Company, Ltd.), and gelatin (Kishida Chemical Company, Ltd.) were used.

Preparation of Immobilized α-Amylase

After α-amylase was dissolved in 0.02 M phosphate buffer solution (pH 6.9) containing HEMA, this solution was poured into a glass ampoule containing the absorbent. The ampoule was sealed and kept overnight at 25°C. The immobilization of α-amylase was carried out with 1×10^6 rads of γ-ray at −24°C. The resultant immobilized α-amylase composite was in a pellet form of 16 mm$\Phi \times 5$ mm.

Preparation of Immobilized Cellulase

Flocking of the enzyme with a natural polymer was carried out by three different methods. The first was the coprecipitation method; that is, cellulase and natural polymers such as gelatin and agar were dissolved and then precipitated with the agents acetone and methanol. The precipitate was isolated and dried, added to 0.1 M acetic acid buffer (pH 4.5) containing HEMA, and quickly cooled to low temperatures in a dispersed state for

immobilization. The second method was the coevaporation approach; that is, a homogeneous aqueous solution of cellulase and the natural polymer was prepared and evaporated by a freeze-drying technique. The remaining residue was added to a buffer solution of HEMA and cooled to low temperatures for immobilization. The third method was the mere coexistent method as a blank system. That is, cellulase, monomer, and natural polymer were mixed in buffer solution at the same time to form the homogeneous aqueous solution and then cooled for immobilization. The immobilization was carried out by irradiating the sample in a glass ampul with γ-ray (1×10^6 rads) at -78°C.

Examination for Leakage of Enzymes from Polymer Matrix

Examination for leakage of α-amylase from the polymer matrix was carried out by repeating the batch enzyme reaction (1.0 h at 40°C). The substrate used was 2 or 10% starch solution in 0.02 M phosphate buffer solution (pH 6.9). The maltose formed was determined by the 3,5-dinitrosalicylic acid method (3). The enzymatic activity of the immobilized α-amylase was determined by maltose formation in the immobilized and the native α-amylase for each batch enzyme reaction.

Examination for leakage of cellulase from the polymer matrix was carried out by repeating the batch enzyme reaction (0.5 h at 40°C). The substrate used was 0.2% CMC buffer solution (0.1 M acetic acid buffer, pH 4.5). The glucose formed was determined using "GOD PODLK" obtained from Nagase Sangyo Company, Ltd. The enzymatic activity of the immobilized cellulase was determined from the glucose formation in the immobilized and the native cellulase for each batch enzyme reaction.

RESULTS AND DISCUSSION

Double Entrapping Immobilization of α-Amylase by Polymerization of HEMA in the Presence of Inorganic Absorbent

The immobilization was carried out by polymerizing the mixture which was prepared by mixing the enzyme–additive adsorption mixture with monomer. The effect of various inorganic additives on the enzymatic activity of immobilized α-amylase is shown in Fig. 1b. It was found that enzymatic activity increased by addition of these additives. Moreover, no decrease of enzymatic activity was observed with each batch enzyme reaction (the repeated use) for every additive. This observation suggested that enzyme leakage was considerably prevented by the double entrapping effect. Drierite was found to be the most effective absorbent of those

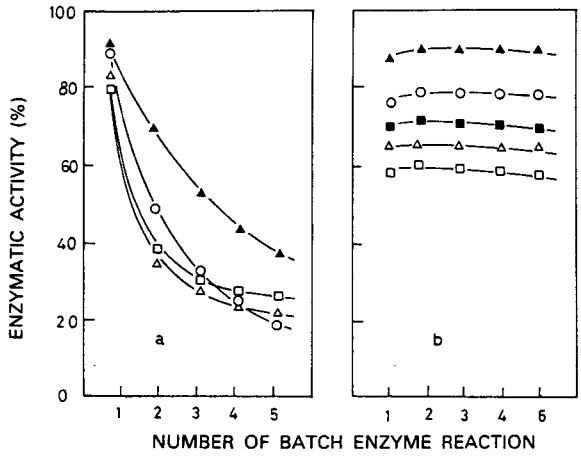

FIG. 1. (a) Adsorption of α-amylase by various additives. α-Amylase concentration 0.2 mg/ml; additive concentration 0.3 g/ml. (b) Effect of various additives on the enzymatic activity of immobilized α-amylase. HEMA concentration 30%; α-amylase concentration 0.2 mg/ml; additive concentration 0.3 g/ml. ○, Silica gel; □, molecular sieves 4A; △, activated carbon; ▲, Drierite.

examined. Figure 1a shows the decrease in enzymatic activity with each batch enzyme reaction using derivatives prepared by adsorbing the enzyme but without polymerization with monomer.

The enzymatic activity decreased rapidly with the repeated batch runs owing to the enzyme leakage in those systems. It was natural to consider that the remarkable immobilization effect observed in Fig. 1b could be attributed not to the mere adsorbing effect by the additive or the trapping effect of the polymer, but to the multiple effect of double entrapping by the additive and the polymer. The effects of the additive, monomer, and enzyme concentrations on the stability of immobilized α-amylase are given in Figs. 2–4. Optimum concentrations of additive, monomer, and enzyme were determined. These preparations showed stable activity and no losses due to enzyme leakage. The quantity 0.3–0.5 g/ml of additive, 30% monomer, and the range 0.2–10 mg/ml enzyme were found to be the optimum. Furthermore, limiting concentrations of these components which permitted enzyme leakage on repeated batch enzyme reactions were observed and could be set as shown in Figs. 5 and 6. In these two figures, enzymatic activity decreases with the repeated batch enzyme reaction due to enzyme leakage under the conditions of region A and no enzyme leakage is observed under the conditions of region B. Enzymatic activity decreases because of enzyme leakage from higher enzyme concentrations

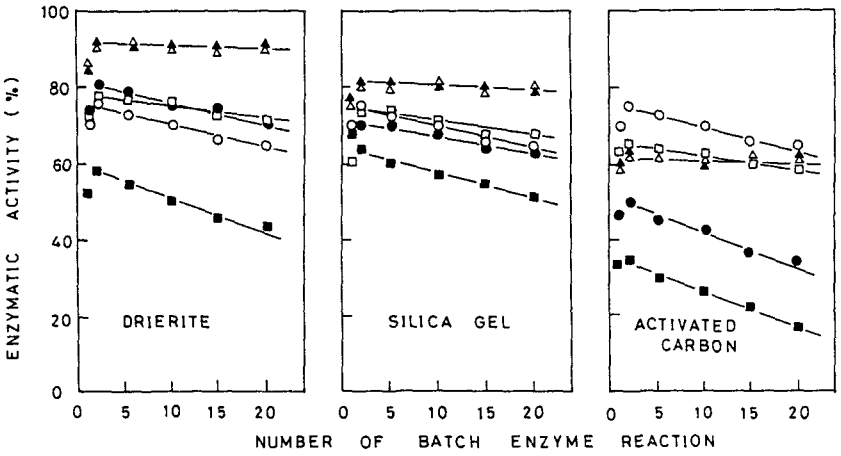

FIG. 2. Effect of additive concentration on the enzymatic activity of immobilized α-amylase. HEMA concentration 30%; α-amylase concentration 0.2 mg/ml; additive concentration (g/ml): ○, none; □, 0.1; △, 0.3; ▲, 0.5; ●, 0.7; ■, 1.0.

and because lower monomer concentration regions in single trapping systems have an absence of additives as shown in Fig. 5. The addition of additives moved the limiting condition curve to the left, thereby enlarging region B. According to Fig. 6, the additive concentration range was enlar-

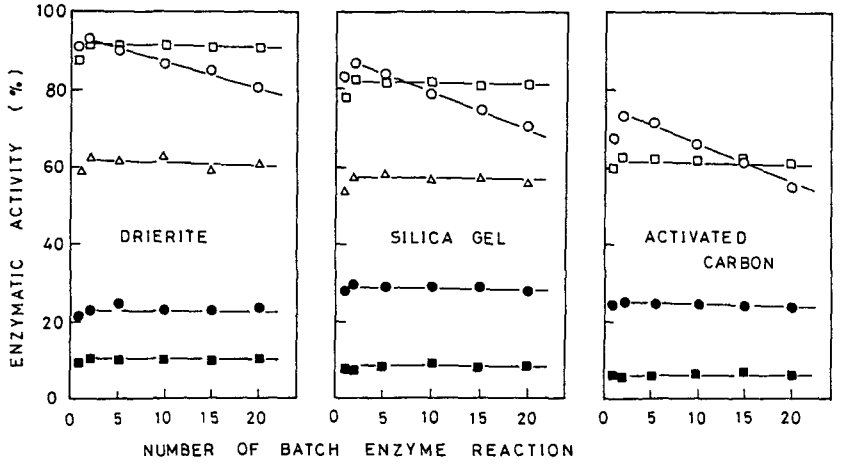

FIG. 3. Effect of HEMA concentration on the enzymatic activity of immobilized α-amylase. α-Amylase concentration 0.2 mg/ml; HEMA concentration (%): ○, 10; □, 30; △, 50; ●, 70; ■, 100.

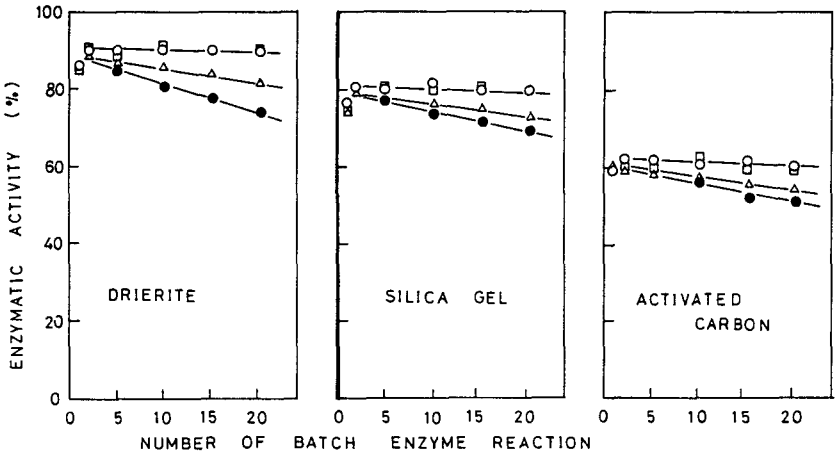

FIG. 4. Effect of α-amylase concentration on the enzymatic activity of immobilized α-amylase. HEMA concentration 30%; additive concentration 0.3 g/ml; α-Amylase concentration (mg/ml): ○, 0.2; □, 10; △, 20; ●, 50.

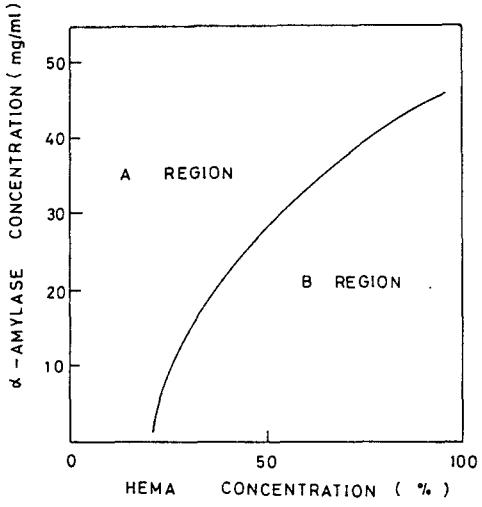

FIG. 5. Entrapping limitation curve of imbolized α-amylase as the function of the enzyme and HEMA concentration. Additive concentration 0.3 g/ml. A region: decrease of enzymatic activity occurred with increase of the reaction number. B region: decrease of the enzymatic activity did not occur with increase of the reaction number.

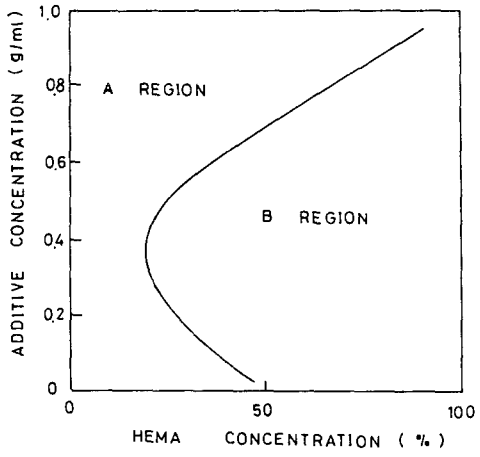

FIG. 6. Entrapping limitation curve of immobilized α-amylase as the function of additive and HEMA concentration. α-Amylase concentration 0.2 mg/ml.

ged as was the no-leakage condition region for the monomer concentration as well as for the enzyme concentration. Excess addition of the additive, however, again narrowed the no-leakage region. Consequently, it could be concluded that double entrapping by adsorbent and by polymer was effective in increasing the efficiency and firmness of the enzyme trapping. It also heightened enzymatic activity and prevented enzyme leakage on repeated use even at lower monomer concentrations and higher enzyme concentrations as compared to the single trapping systems which do not include additives.

Double Entrapping Immobilization of Cellulase by Polymerization of HEMA with a Natural Polymer

Another double entrapping method was studied. This consisted of polymerizing a mixture prepared by mixing the enzyme–natural polymer mixture with monomer in the buffer solution. The enzymatic activity of the immobilized cellulases obtained by various methods versus the number of batch enzyme reactions is shown in Figs. 7–10. The immobilized enzymes obtained by the coprecipitation and coevaporation methods showed the clear increased stability of enzymatic activity and decreased enzyme leakage on repeated use. On the other hand, mere coexistence or comixing of

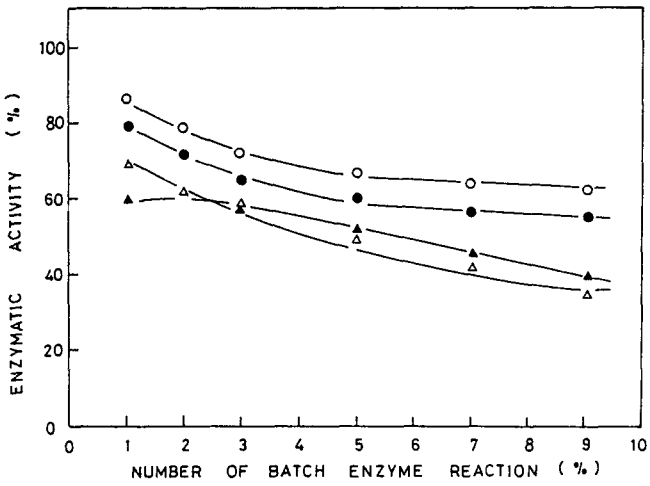

FIG. 7. Effect of double entrapping with gelatin on the enzymatic activity of immobilized cellulase at 30% HEMA concentration. Cellulase concentration: cellulase/additive = 10%; entrapping method: ○, coprecipitation; ●, coevaporation; △, comixing; ▲, single entrapping.

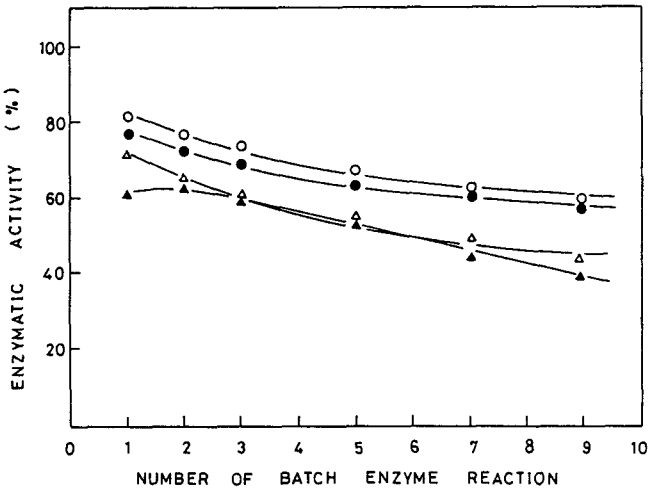

FIG. 8. Effect of double entrapping with agar on the enzymatic activity of immobilized cellulase at 30% HEMA concentration. Cellulase concentration: cellulase/additive = 10%; entrapping method: ○, coprecipitation; ●, coevaporation; △, comixing; ▲, single entrapping.

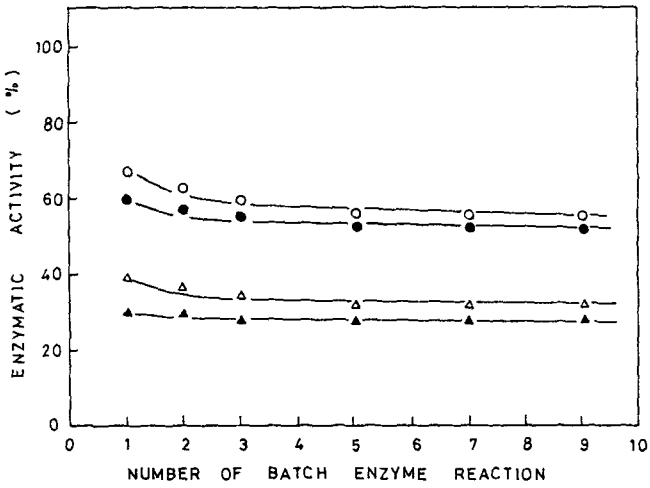

FIG. 9. Effect of double entrapping with gelatin on the enzymatic activity of immobilized cellulase at 70% HEMA concentration. Cellulase concentration: cellulase/additive = 10%; entrapping method: ○, coprecipitation; ●, coevaporation; △, comixing; ▲, single entrapping.

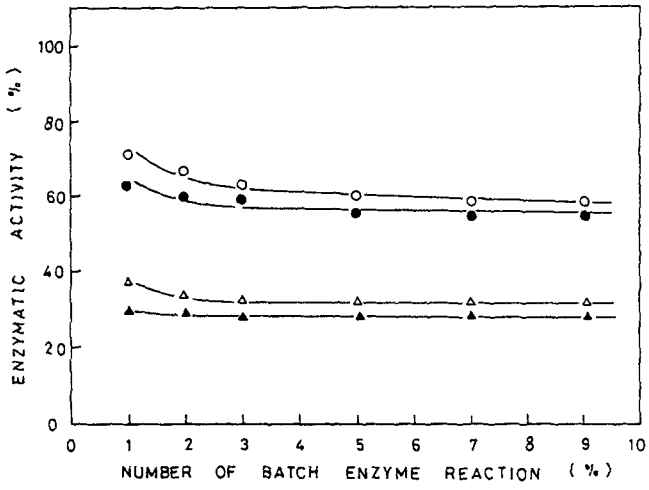

FIG. 10. Effect of double entrapping with agar on the enzymatic activity of immobilized cellulase at 70% HEMA concentration. Cellulase concentration: cellulase/additive = 10%; entrapping method: ○, coprecipitation; ●, coevaporation; △, comixing; ▲, single entrapping.

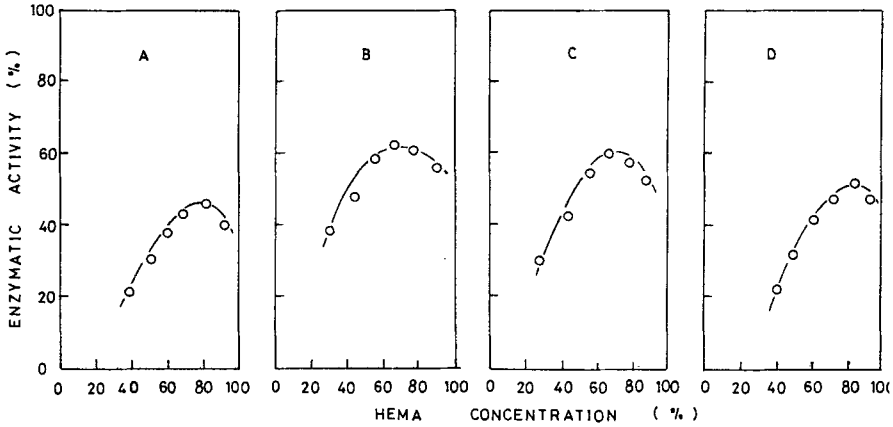

FIG. 11. Relation between HEMA concentration and the enzymatic activity of immobilized cellulase by various entrapping methods using agar. Cellulase concentration: cellulase/additive = 10%; entrapping method: (A) single entrapping; (B) coprecipitation; (C) coevaporation; (D) comixing.

the reagents gave improvement in enzymatic activity over the controls. Preliminary entrapping or aggregating of enzyme and natural polymer molecules was necessary for realization of the desired effect. These results support the idea of double entrapping by natural polymer and also by carrier polymer.

The relation between monomer concentration and enzymatic activity of immobilized cellulase by various double entrapping methods is shown in Fig. 11. The enzymatic activities of coprecipitation and coevaporation systems were greater at higher monomer concentrations by at least 10% than the enzymatic activities observed in the mixed and no-additive systems. In general, gelatin as a natural polymer was preferable to agar. The effect of additive concentration and the conditions for coprecipitation have not yet been clarified in detail; these will be studied in the future.

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