Porous Substances Immobilizing Enzymes with Polymer Matrix

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Synopsis

The preparation of porous substances immobilizing enzymes with polymer matrix having various properties have been studied by radiation polymerization method. The enzyme (cellulase) was immobilized on the surface parts of porous substances such as activated carbon, molecular sieve, silica gel, and coating with hydrophilic and hydrophobic monomers. As substrate of enzyme reaction, lignocellulosic wastes such as chaff pretreated by radiation irradiation (100 Mrad) were used. The enzyme activity of the immobilized enzyme substances was markedly affected by hydrophilicity of monomer and the copolymerization of methoxypoly(ethyle glycol) methacrylate (75%) and poly(ethylene glycol) dimethacrylate (25%) monomer gave the most highest enzyme activity. The immobilized enzyme substances was able to hydrolyze chaff pretreated by radiation irradiation.

INTRODUCTION

Inorganic materials such as activated carbon, silica gel, etc., have been used as adsorption reagent in various fields. The immobilization of enzymes using silica gel has been studied by some workers utilizing its adsorption property.[1-3] The high sensitivity of enzymes toward action of external factors and especially the difficulties encountered in catalyst regeneration have restrained large scale applications. The loss of enzyme activity is determined by the change of intramolecular interactions leading to the modification of enzyme molecular architecture. The preservation of the degree of enzyme activity is a direct quantitative measure of enzyme stabilization. The physical and/or chemical immobilization on solid supports is the usual way for enzyme stabilization, which has opened new possibilities to their efficient applications in the fields of biotechnology, medical, and chemical processes.[4-7] The complexity of enzyme immobilization process make their research yield yet an empirical one. The practical applications of immobilized enzymes outdistanced the knowledge mechanism of the stabilization process, becoming a yield of great economic interest. We have studied the immobilization of biological substances such as enzymes, antibodies, and cells by radiation polymerization.[8-10] In this work, the immobilization of enzymes by radiation polymerization using porous substances such as silica gel and activated carbon has been studied.

MATERIALS AND METHODS

Monomers

Hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), trimethylpropane triacrylate (TMPT), poly(ethylene glycol) dimeth-

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acrylate [$CH_2C(CH_3)COO(CH_2CH_2O)_nOC(CH_3)CCH_2$, nG], methoxynonaethyleneglycol methacrylate (M-9G), and methoxypolyethyleneglycol methacrylate [$CH_2C(CH_3)COO(CH_2CH_2O)_{23}CH_3$, M-23G] were used as monomer, which were obtained from Shin Nakamura Chemical Co., Ltd.

Enzyme and Porous Substances

Cellulase (EC 3.2.1.4. from Trichoderma viride) was obtained from Yakult Biochemicals Co. Ltd., its activity being 1×10^4 units/g. Silica gel (Blue, 2 mmϕ, from Kanto Chemical Co., Ltd.), activated carbon (granular, 2 mmϕ, from Wako Pure Chemical Industries, Ltd.), molecular sieve (4A, type 1/16, 2 mmϕ × 5 mm, from Nishio Industries Co., Ltd.), poly(methyl methacryalte) (2 mmϕ × 3 mm, from Wako Pure Chemical Industries Ltd.), and polystylene (2 mmϕ × 3 mm, from Kishida Chemical Co., Ltd.) were used as base substances.

Preparation of Immobilized Enzyme Substances

Two milliliters of the enzyme solution (enzyme, 20 mg; monomer, 1.6 mL) containing 0.1M acetate buffer, pH 4.5, were coated on the surface of the base substances (10 g) by agitating in a vessel. The coated substances were irradiated with irradiation dose of 1 Mrad by γ-rays from ^{60}Co source at $-78°C$. After irradiation, the immobilized enzyme substances obtained were washed with 0.1M acetate buffer solution and dried at room temperature.

Radiation Treatment of Chaff

As substrate in enzyme reaction using the immobilized enzyme substances, chaff pretreated by radiation irradiation was used. Chaff was put into a plastic bag and irradiated with a Dynamitron IEA-3000-25-2 electron beam accelerator. Chaff sample (thickness 1–1.5 cm) was irradiated in an air atmosphere using belt-conveyer irradiation equipment where the sample supporter on the belt used a metal plate (aluminum, 0.5 cm in thickness). The distance between the electron beam window and the surface of the belt conveyer was fixed at 2.26 m/min. The irradiation with various irradiation doses was performed by the repetition of the belt conveyer in a certain stroke, in which the irradiation of one stroke in the condition of 2 MeV electron beam accelerator voltage and 5 mA electron beam current gave an irradiation dose of 5 Mrad. After irradiation, the samples were crushed with a mechanical crushing machine (Turbo-Mill T-250).

Batch Enzyme Reaction with Immobilized Enzyme Substances

To examine the durability of the immobilized enzyme substances, the enzyme activity of the immobilized enzyme substances was followed up by repeating batch enzyme reaction, in which one batch enzyme reaction was carried out at 40°C for 1 day using chaff pretreated by radiation irradiation (100 Mrad) as substrate, the substrate concentration being 20%. In the batch enzyme reaction, 10 g of the immobilized enzyme substances and 20 mL of the substrate solution (pH 4.5) were put into a vessel (100 mL) and shaken in a

water bath (40°C) using a shaker during the enzyme reaction. After each batch enzyme reaction, the substrate solution was changed by new substrate solution (20 mL). Glucose formed in the batch enzyme reaction was measured with a glucose analyzer (CGA-101 Shimazu glucose analyzer). The enzyme activity (%) remaining in the repeated batch enzyme reaction was obtained from the glucose formation ratio of the immobilized and native enzyme (enzyme, 20 mg) at each batch enzyme reaction.

RESULTS AND DISCUSSION

Coating of Enzyme on Various Substances

The enzyme was coated on substances such as poly(methyl methacrylate) (PMMA), polystyrene (PST), activated carbon, silica gel, and molecular sieve using tetradecaethyleneglycol dimethacrylate (14G) monomer by radiation polymerization. The relationship between enzyme activity and number of repeat batch enzyme reaction is shown in Figure 1. The enzyme activities of the immobilized enzyme substances with the substances of PMMA and PST decreased gradually with increasing number of batch enzyme reaction. On the other hand, the enzyme activities of the immobilized enzyme substances with the substances of activated carbon, molecular sieve, and silica gel were constant with repeated batch enzyme reactions, though the enzyme activity of the immobilized enzyme substances with activated carbon increased at initial stage with repeated batch enzyme reactions. The substances such as PMMA and PST are a polymer particle, of which the porous structure is not

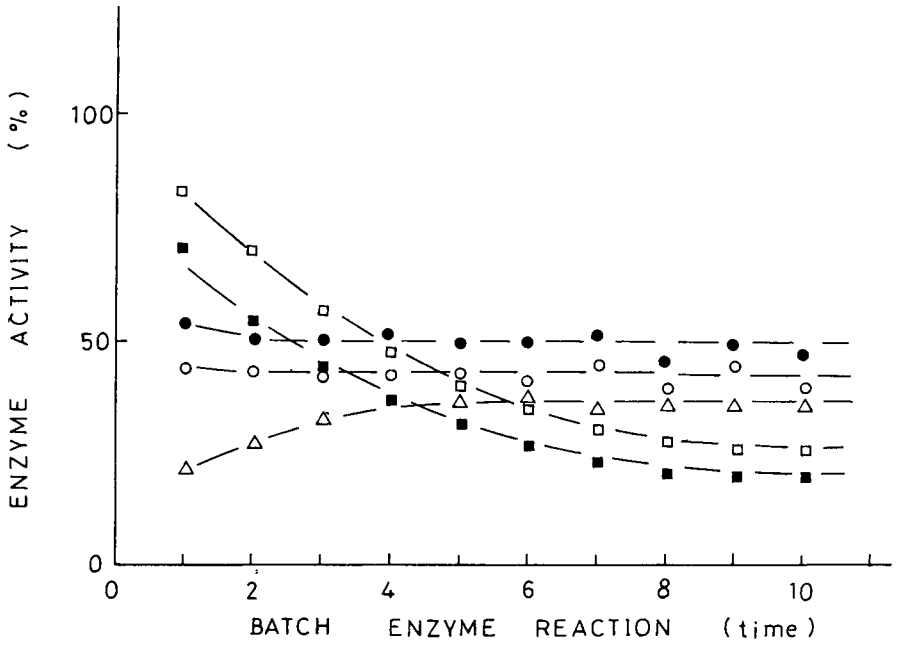

Fig. 1. Relationship between enzyme activity and number of repeated batch enzyme reaction in the immobilized enzyme substances obtained by using 14G monomer and various porous substances: (△) activated carbon; (○) molecular sieve; (●) silica gel; (■) PMMA; (□) PST.

contained. From the result of a reasonable high enzyme activity in the immobilized enzyme substances with porous substances, it is though that low enzyme activity in the immobilized enzyme substances with PMMA and PST is due to less porous structure. The enzyme is, of course, immobilized on the surface part consisting of 14G polymer in the immobilized enzyme substances with PMMA and PST. However, the connection between 14G polymer layer and the substance seems to be imperfect by which the enzymes might be leaked. The decrease of the enzyme activity with repeated batch enzyme reaction means that a leakage of the enzymes occurs, whereas the increase of the enzyme activity in the case of activated carbon means that the enzymes are entrapped in the inside of the activated carbon particle and thereby the diffusion of the substrate takes the rate determining step in the enzyme reaction.

The result of the immobilization of the enzymes using HEMA monomer instead of 14G monomer is shown in Figure 2. The tendency of the enzyme activity with repeated batch enzyme reaction in the porous substances of activated carbon, molecular sieve, and silica gel was similar to that in 14G monomer. However, though the entire enzyme activity in PMMA and PST decreases at the first stage, it became constant at the later stage, indicating that the leakage of the enzymes stops after repeated batch enzyme reactions of two or three times. The polymer matrix obtained from HEMA monomer being monomethacrylate molecular structure give a gel-like state in which the enzymes are conveniently trapped on the surface of the polymer matrix and are able to react with the substrate. But the polymer matrix from 14G

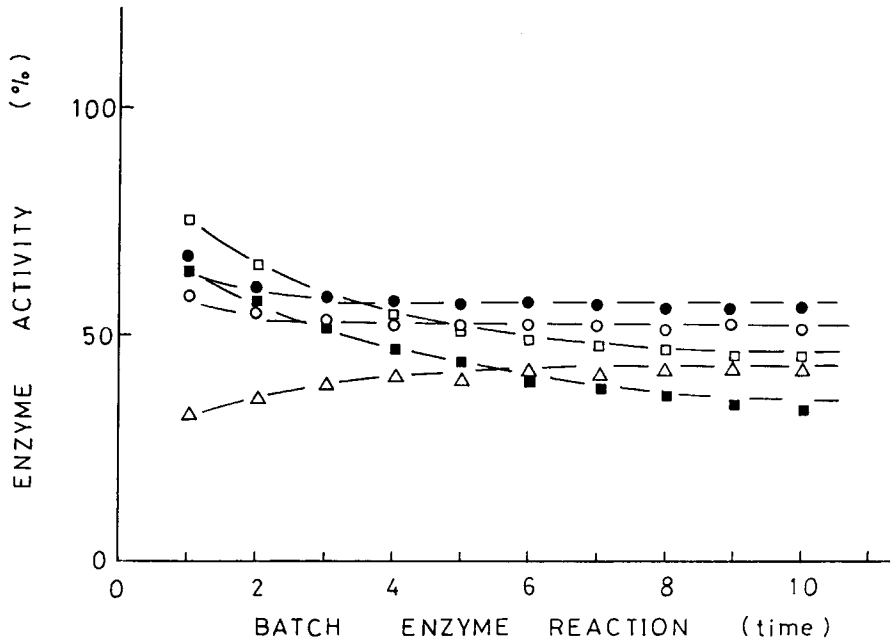

Fig. 2. Relationship between enzyme activity and number of repeated batch enzyme reaction in the immobilized enzyme substances obtained by using HEMA monomer and various substances: (△) activated carbon; (○) molecular sieve; (●) silica gel; (■) PMMA; (⊙) PST.

monomer, which is dimethacrylate molecular structure, having bifunctional group, gives a porous hard state and is fragile. Thus, the behavior of the enzyme activity of the enzymes immobilized in the polymer matrix was affected by the nature of the polymer matrix and the porous substance. The polymerization of monomer on the surface of the porous substance converting to polymer matrix was carried out by radiation reaction at low temperature (−78°C) by which the enzymes were immobilized in the polymer matrix with the porous substance keeping the initial activity of the enzymes without radiation damage because of low temperature.

Effect of Hydrophilicity on Enzyme Activity

The enzymes were immobilized on silica gel using various monomers to examine the effect of the property of monomer as shown in Figure 3. In Figure 3, the enzyme activities in M-23G and M-9G monomers were relatively high and constant with repeated batch enzyme reaction, but that in TMPT was decreased. The polymer matrix obtained from M-23G and M-9G monomers having hydrophilic property gave a soft gel-like state and its softness was higher than that of HEMA monomer, by which the enzymes would be mildly immobilized in their polymer matrix rather than HEMA polymer matrix, allowing more mobility of the enzyme molecule. The enzyme activity of the immobilized enzyme substances obtained from M-23G monomer appeared to be higher than that from M-9G monomer, the hydrophilicity of polymers from methoxypolyethyleneglycol methacrylate monomers having long oxyethylene unit chain increased with increasing oxyethylene chain length, that is, the

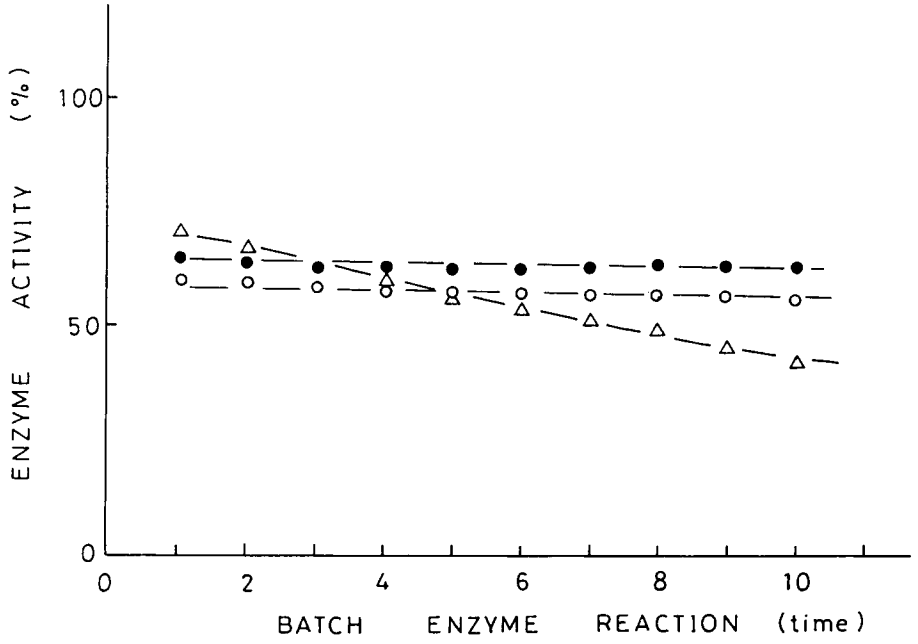

Fig. 3. Relationship between enzyme activity and number of repeated batch enzyme reaction in the immobilized enzyme substances obtained by using silica gel and various monomers: (●) M-23G monomer; (○) M-9G monomer; (△) TMPT monomer.

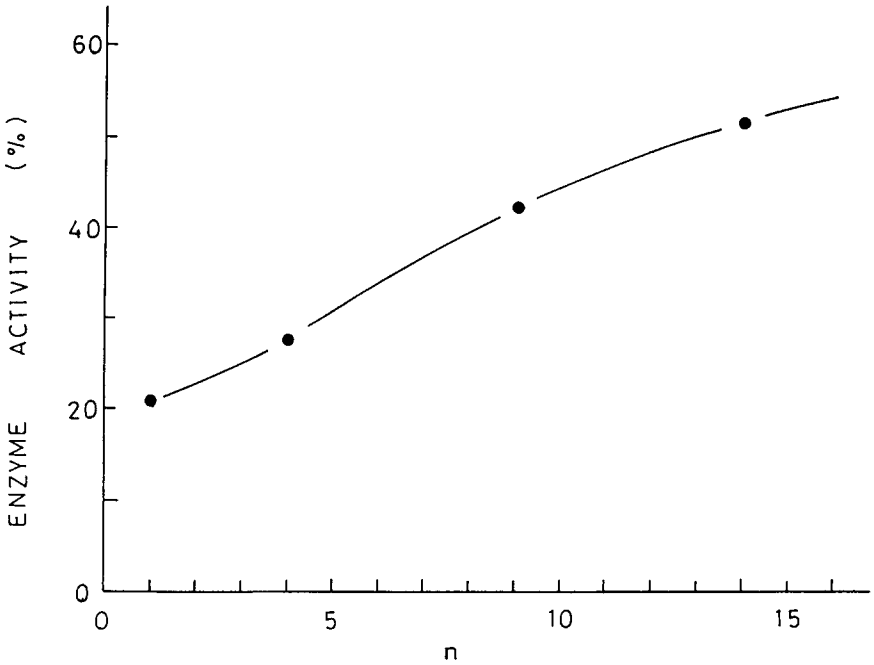

Fig. 4. Effect of number (n) of oxyethylene unit in poly(ethylene glycol) dimethacrylate (nG) monomers on enzyme activity; porous substance, silica gel.

hydrophilicity of the polymer matrix from M-23G monomer was higher than that from M-9G monomer. Water contents of M-23G and M-9G polymer were 87 and 76%, respectively. The polymer matrix from TMPT monomer, which is a hydrophobic trifunctional one (water content of TMPT polymer was 5%), was a hard state and was able to swell in the substrate to the enzymes immobilized in the polymer matrix was low, affecting the enzyme activity. The enzyme activity in TMPT monomer decreased, thus, with repeated batch enzyme reaction due to the limitation of the enzyme reaction, as shown in Figure 3.

The nG monomer having oxyethylene units were used as monomer for immobilization, and the relationship between enzyme activity and number of oxyethylene unit in the monomers is shown in Figure 4. The enzyme activity increased with increasing number of oxyethylene unit in which the hydrophilicity of the resultant polymer matrix increased with increasing number of oxyethylene unit, that is, the water content of 14G monomer was 23%. The immobilized enzyme substances obtained by using these nG monomers were hard.

Effect of Copolymerization on Enzyme Activity

The enzymes were immobilized by copolymerization of hydrophilic M-23G monomer and hydrophobic TMPT monomer coated on silica gel, and the relationship between enzyme activity and monomer composition was examined as shown in Figure 5. The enzyme activity had a maximum point at a certain monomer composition, showing that optimum condition of the poly-

mer matrix for immobilization exists. As mentioned above, the polymer matrix from M-23G monomer was very soft gel as if the polymer matrix layer is torn from silica gel surface. On the other hand, the polymer matrix from TMPT monomer was rigid in which the enzyme reaction was carried out only the surface of the polymer matrix. The polymer matrix obtained from the monomer composition of 75% M-23G monomer and 25% TMPT monomer, giving a maximum enzyme activity, was a suitable mechanical strength resulting from the formation of crosslinked polymer due to the addition of the trifunctional monomer by which the polymer matrix was not torn from silica gel; consequently, the enzymes were firmly trapped in the polymer matrix to be of available for the enzyme reaction. The substrate used in this work was chaff powder pretreated by radiation irradiation and subsequent mechanical crushing, and the tissue of chaff was perfectly degraded and the cellulose part was located on the surface of the powder to be able to react with the enzymes immobilized on the polymer matrix. Though lignocellulosic wastes such as chaff include hemicellulose, lignin, and silicates, except for cellulose, keeping physical bonding to each other, the bonds of these components could be broken up by radiation irradiation of 100 Mrad, and most of the components come to be dissolved in water, resulting in a decrease of polymerization degree. As can be seen in Figures 1–5, the immobilized enzyme substances obtained in this immobilization method had the enzyme activity of 50–80%. This indicates that the pretreated chaff is efficiently hydrolyzed by the immobilized

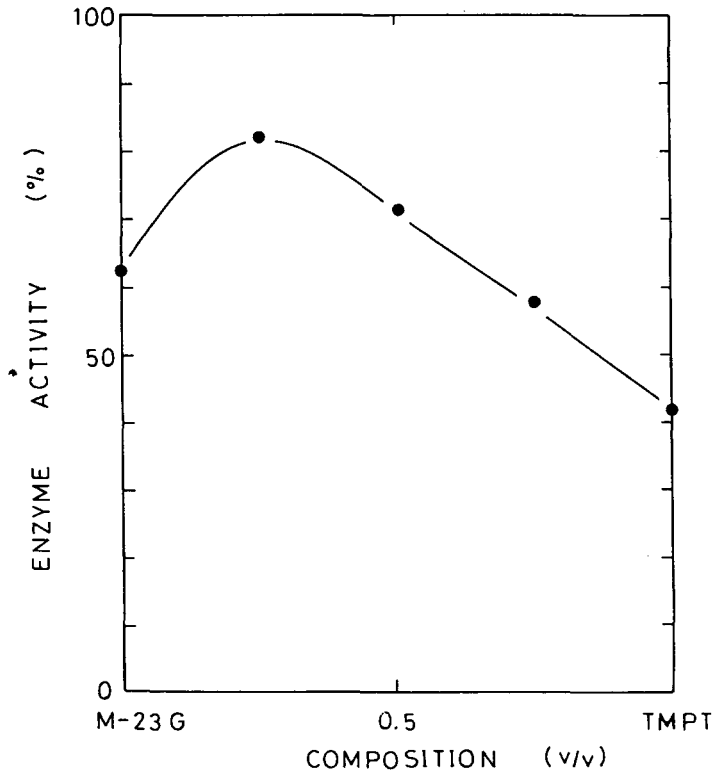

Fig. 5. Effect of copolymerization on enzyme activity; porous substance, silica gel.

enzymes to produce glucose and that the immobilized enzyme substances obtained by this method are able to be used for the hydrolysis of lignocellulosic wastes.

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