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PREPARATION AND USE OF POLYURETHANE ENZYME PARTICLES

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Abstract

The preparation and use of immobilized enzyme particles by dispersion and radiation polymerization technique in polyurethane prepolymer using tolylene-2,4-diisocyanate, 2-hydroxyethyl methacrylate, pentamethylene glycol, and cellulase are presented. The enzyme was added to form the particles (10 - 1000 μm in diameter) under stirring of the mixture containing the prepolymer and enzyme solution, in which the enzyme was covalently bound to the surface of the particle. The size of the particles varied with the concentration of the enzyme and monomer. The enzymatic activity of the particles varied with the particle diameter, composition of monomer, and dispersion temperature.

Introduction

Enzymes which are highly efficient and specific biological catalysts generally function in the soluble

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form. Since they are difficult and costly to isolate in a reasonably pure form, their use in industrial processes is restricted, since once used, they cannot be conveniently recovered. To overcome these limitations, various techniques have been developed to render enzymes water-insoluble.[1-3] Among immobilized enzymes in various forms, immobilized enzymes in particle form are very useful for column reaction in the continuous mode. Therefore, some attempts have been made to obtain immobilized enzyme particles by using glass beads[4], styrene,[5] acrylamide,[6] and acrylic monomers[7] via methods of chemical bonding and entrapping. The immobilization of enzyme using metal-activated controlled pore glass was studied by Cabral et al.[8] and the stabilities of the immobilized enzyme preparations obtained without and with a crosslinking step by means of glutaraldehyde and tannic acid were compared. Covalent binding to polymeric carriers has as yet been the most thoroughly studied procedure of the immobilization of enzymes. With the exception of very few cases, the composition and structure of protein molecules does not allow to formulate general rules of choice of an adequate polymeric carrier. Valentova et al.[9] have studied the choice of a suitable type of binding reaction for the immobilization of the glycoprotein enzyme using carriers based on glycidylmethacrylate and cellulose beads. At present immobilization of enzymes has been carried out frequently on inorganic and organic beads. A number of papers have appeared recently concerning immobilization of enzymes, mostly on glass beads.[4] However, these immobilization methods need various chemical agents to bind enzymes to the beads. Many of the hitherto developed immobilized enzyme particles necessitated long preparation times, taking two steps for the formation of particles and the binding of enzymes.

In this work, a new preparation method for immobilized particles was studied using tolylene-2,4-diisocyanate, in which the preparation time was short and stabilizing reagents for the particles were not necessary. The present method makes it possible to obtain immobilized enzyme particles by a simple process, involving the formation of the particles and the binding of enzymes.

Experimental

1. Materials

Tolylene-2,4-diisocyanate (TDI), pentamethylene glycol (PG), and 2-hydroxyethyl methacrylate (HEMA) were obtained from Wako Pure Chemical Industries, Ltd. Cellulase was used as enzyme for immobilization and obtained from Yakult Mfg Co., Ltd. Albumin (from eggs; Kishida Chemical Co., Ltd.), polyoxyethylene sorbitan monostearate (Tween 60; Tokyo Kasei Kogyo Co., Ltd.), polyvinylpyrrolidone (M=10000; Kishida Chemical Co., Ltd.) and sodium dodecyl sulfate (SDS; Pierce Chemical Company) were used as dispersion agents.

2. Preparation of immobilized enzyme particles

2.1. Dispersion technique in polyurethane prepolymer using TDI and HEMA(or PG)

TDI and HEMA were put into a flask and mixed. The TDI and HEMA were slightly reacted for 1 - 5 min, after which the viscosity of the mixture had slightly increased. An aqueous solution of enzyme was added to the mixture and quickly agitated with a stirrer for 5 min. After agitating, polyurethane - enzyme particles were obtained.

2.2. Radiation polymerization technique in dispersed polyurethane prepolymer using TDI and HEMA

TDI and HEMA were mixed, and allowed to react for

1 - 5 min, until the viscosity of the mixture had slightly increased. 0.1 M acetate buffer solution, pH 4.5 containing the enzyme was added to the mixture, in which the enzyme concentration was 0.5%, and stirred vigorously for 30 min. The flask was then cooled immediately to -78°C. The γ-ray irradiation (1×10^6R) of the flask was carried out at -78°C for 1 hr. After irradiation, immobilized enzyme particles were obtained by warming the mixture to room temperature.

3. Enzymatic activity of immobilized enzyme particles

The durability of the enzymatic activity of immobilized enzyme particles was examined by repeating the batch enzyme reaction (1.0 hr at 40 °C, pH 4.5). The enzymatic activity (%) was obtained from the glucose formation ratio in immobilized enzyme particles and native enzymes with each batch of enzyme reaction. A 1.0% carboxymethylcellulose sodium (CMC) solution containing the acetate buffer solution was used as the substrate. The glucose was measured with glucose specific reagent, "COD-PODLK", obtained from Nagase Sangyo Co., Ltd.

Results and Discussion

1. Dispersion of polyurethane prepolymer in TDI - HEMA system

The stability of the particles formed by the dispersion of polyurethane prepolymer was studied using proteins and surfactants. In general, the polyurethane is formed by the known reaction of di- and tri-isocyanates and other polyisocyanates with compounds containing active hydrogen such as glycols, polyglycols, polyester polyols, et al. This reaction makes polyurethane polymers. In the present method, it is

a key point to make a stable particle from polyurethane prepolymer using proteins such as enzymes. TDI reacted easily with HEMA to form polyurethane at room temperature. The aqueous solution containing proteins or surfactants was added to the mixture of TDI and HEMA before the polyurethane was perfectly formed. The proteins, such as enzyme and albumin, were reacted to form the particles in the reaction mixture. However, particles were not formed with dispersion agents (surfactants) such as Tween 60, SDS, and polyvinylpyrolidone. In the presence of these dispersion at various concentrations (0 - 2%) and at 10% monomer concentration, the polyurethane prepolymer was in bulk form rather than particle form after mixing the system. Thus, the particles resulting from the dispersion of polyurethane prepolymer appeared to have reacted with proteins. The formation mechanism of the particles in the present method was as follows; TDI reacts slightly with HEMA having a hydroxyl group and gives a polyurethane prepolymer, most of which have isocyanate groups remaining. Then, the polyurethane prepolymer which is added to the aqueous solution containing protein by agitating or stirring, forms immobilized protein particles, in which the dispersion and the binding (immobilizing) takes place at the same time. In the present method, the polyurethane prepolymer appeared to be dispersed by covalent binding of the proteins. Therefore, when using agents such as Tween 60, SDS, and polyvinylpyrolidone instead of enzyme, the particles were not formed covalently with the polyurethane prepolymer. The polyurethane prepolymer is viscous and has hydrophilic properties, and the molecular cohesion energy is very large, 8.74 kcal/mol.[10] Therefore, the polyurethane prepolymer in aqueous solution could not be dispersed by the usual dispersion

agents, and rather formed a polyurethane foam-like polymer by the reaction with water. The polyurethane prepolymer has a higher reactivity for proteins having amino groups than for other compounds such as water. In general, it is known that the order of the reactivity of isocyanate groups with other compounds is as follows; $RNH_2 > R_2NH > RCH_2OH > H_2O > RCOOH$.[11] The formation of immobilized enzyme particles in the present method is based on this difference of reactivity. Of course, the proteins such as enzymes and albumin could play a part as dispersion agents also. Thus, the polyurethane prepolymer in the aqueous solution containing the proteins was dispersed to bind initially more to proteins than to water under agitation.

2. Effect of protein concentration on particle size in TDI - HEMA system

The variation of the particle size of the polyurethane-bound proteins with various dispersion conditions was studied. The relationship between particle diameter and the concentration of the proteins is shown in Fig.1. The particle diameter decreased and then became almost constant with increasing the concentration of cellulase or albumin. At low protein concentrations (below 0.05%), the particles were unstable and large particle sizes. On the other hand, at high protein concentrations (above 0.3%), the particle diameter appeared constant within experimental error. As can be seen in Fig.1, the difference in particle size was a function of the difference in the kind of the proteins, the particle diameter in the presence of albumin being smaller than that in cellulase. Since the molecular weight of both albumin and cellulase are comparable, 40000 - 60000,[12,13] the

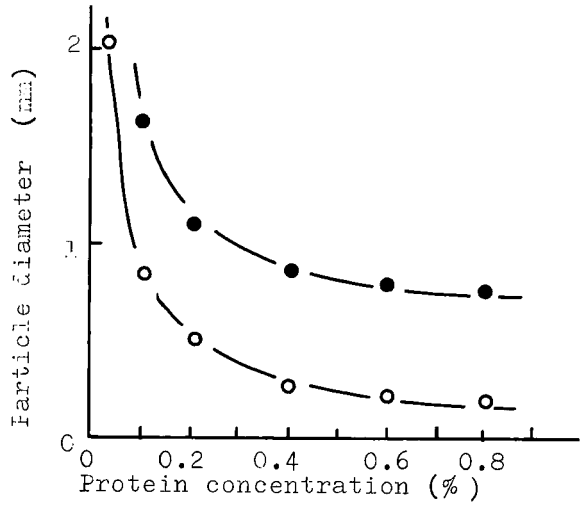


Fig.1 Effect of protein concentration on particle diameter. Monomer (TDI , HEMA) concentration; 10%(V/V), TDI/HEMA=1/1, protein; ● cellulase, ○ albumin

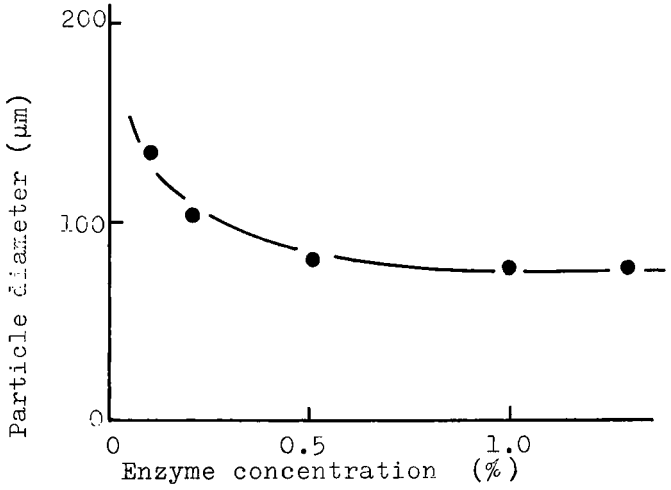


Fig.2 Relationship between particle diameter and enzyme concentration. TDI/PG=1/1; monomer concentration, 10%(V/V)

difference in the particle diameter in Fig.1 seemed to be due to the molecular structure of the proteins.

The effect of enzyme concentration on the size of the particles in TDI - PG system was examined. Fig.2 shows the relationship between particle diameter and enzyme concentration. As the enzyme concentration was increased, the particle diameter decreased markedly at low enzyme concentrations and then became constant. At low enzyme concentrations below 0.05%, the particles were unstable. As enzyme concentration increased, particles with a diameter of 80 μm were formed in the absence of a dispersing agent. This value of the particle size was smaller than that of the particle size in TDI - HEMA system.

3. Effect of monomer concentration on particle size in TDI - HEMA (or PG) system

The relationship between particle diameter and monomer concentration is shown in Fig.3. The particle diameter increased with increasing monomer concentration. At high monomer concentrations above ca. 30%, the formation of small particles was impossible, because of the coagulation of the particles. It is considered that this limitation of the formation of stable particles varied with the concentration of the enzyme. The results in Fig.3 suggest that the surface of the particles with the diameter of ca. 1 mm is covered by the enzymes in large quantity, which overcomes the molecular cohesion energy of the polyurethane prepolymer. The immobilized enzyme particles which were immediately formed by the dispersion were in a relatively soft state, and became gradually more rigid during the polymerization, which inhibited the urethane-binding reaction occurring through the migration of the enzyme and water to the internal part of the particles. Thus, although the immobilized

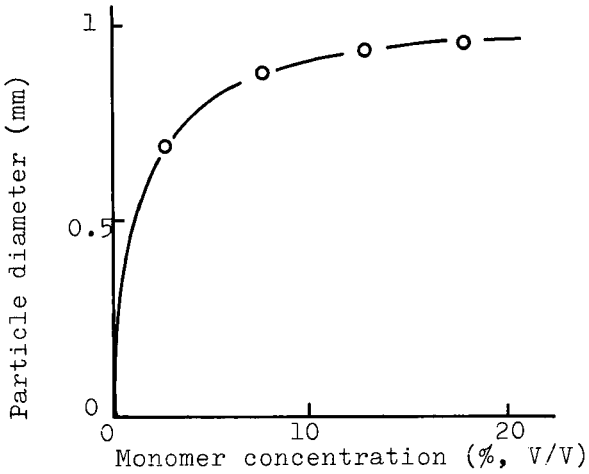


Fig.3 Relationship between monomer concentration and particle diameter. TDI/HEMA=1/1; enzyme concentration; 0.5%

enzyme particles can be prepared by the dispersion technique in TDI - HEMA system, the combined preparation of the dispersion and radiation polymerization techniques was studied to perfectly polymerize the vinyl group in HEMA monomer as described in a later section.

The effect of monomer composition on particle size in TDI - PG system was studied. The particle diameter increased and then became constant with increasing PG component as shown in Fig.4. The polymers from the reaction of TDI with the enzyme solution gave a bulk powder, polymers with a high concentration of the PG component gave a polyurethane sponge-like state. Thus, a 1:1 composition of the TDI - PG system appeared to be suitable for the formation of the particles, showing that one isocyanate group of TDI reacts with one hydroxyl group of PG though a part of PG is reacted

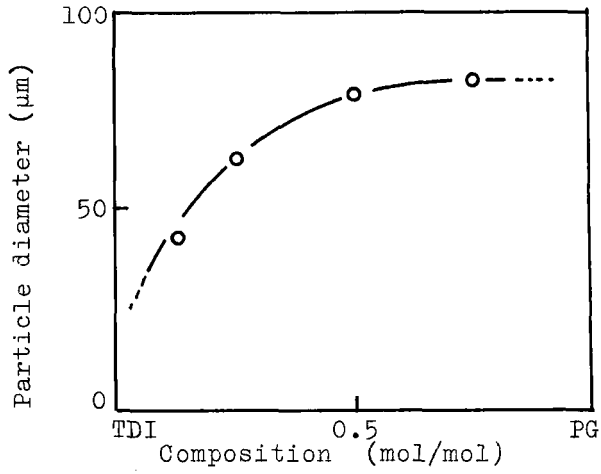


Fig.4 Relationship between particle diameter and monomer composition. Monomer concentration, 10%(V/V); enzyme concentration, 1.0%

with TDI to form polyurethane prepolymer. The decrease of the size of the particle by decrease of PG component is due to the decrease of the formation of the polyurethane prepolymer resulting from the reaction of TDI with PG. The relationship between particle and monomer (TDI and PG) concentration at a 1:1 composition is shown in Fig.5. The size of the particles increased markedly till about 10% monomer concentration and after that increased moderately with increasing monomer concentration. The formation of discrete particles at high monomer concentrations above 40% was impossible because coagulation of the particles then takes place. But, the formation of the particles at high monomer concentrations could be possible by increasing the reaction time.

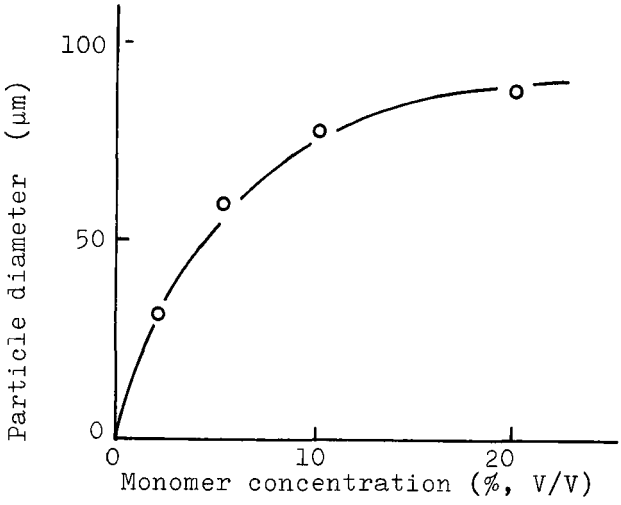


Fig.5 Relationship between particle diameter and monomer concentration. TDI/PG=1/1; enzyme concentration, 1.0%

4. Variation of enzymatic activity with dispersion conditions

The variation of the enzymatic activity of immobilized enzyme particles with dispersion conditions in TDI - HEMA system was studied. The effect of the dispersion temperature on the enzymatic activity was examined as a function of batch enzyme reaction as shown in Fig.6. The enzymatic activity decreased with increasing dispersion temperature, indicating that the enzyme was deactivated by the increase of temperature. From this result, it appears that the dispersion operation in the preparation of immobilized enzyme particles should be carried out at as low temperature as possible. As can be seen in Fig.6, enzymatic

activity did not vary with repeated batch enzyme reactions, indicating that leakage of the enzyme from the particles does not take place because the enzymes are covalently bound to the surface of the particles.

The enzymatic activity varied also with the composition of monomer as shown in Fig.7. The maximum of the enzymatic activity was observed at the composition slightly rich in TDI rather than at an equivalent composition (1:1), indicating that one isocyanate group of TDI reacts equivalently with HEMA and the other isocyanate group of TDI reacts with the enzyme. Increasing the concentration of HEMA in the prepolymer results in decrease of enzymatic activity owing to the absence of the binding site (isocyanate group). Thus, it was found that a composition rich in TDI is suitable for the formation of immobilized enzyme particles which have high enzymatic activity.

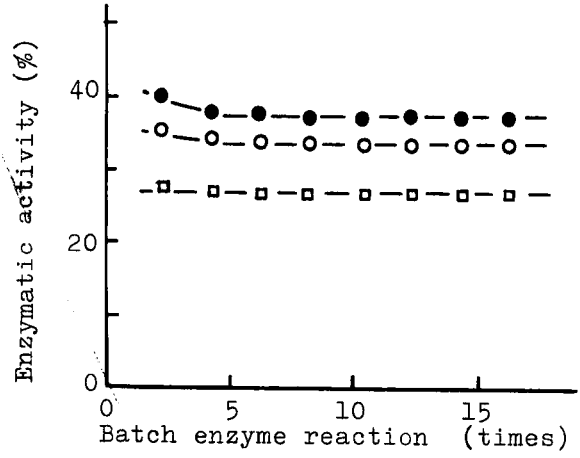


Fig.6 Variation of enzymatic activity with repeated batch enzyme reaction. Monomer concentration; 20%(V/V), dispersion temperature; ● 4°C, ○ 10°C, □ 25°C

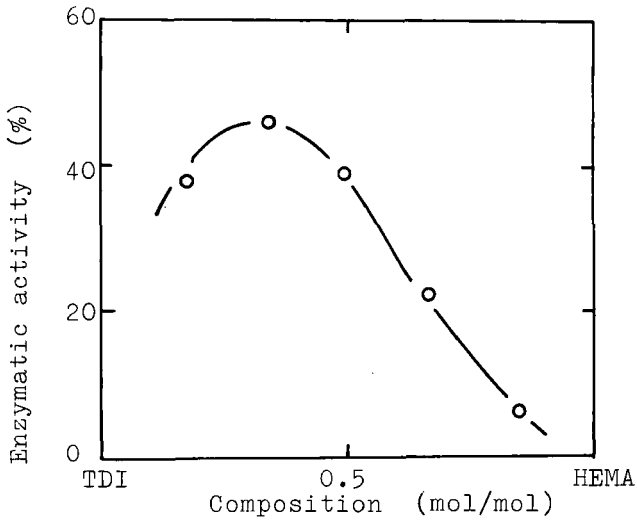


Fig.7 Relationship between enzymatic activity and monomer composition. Monomer concentration; 10%(V/V)

5. Effect of irradiation temperature on enzyme in dispersion and radiation polymerization technique

The immobilized enzyme particles were prepared with the dispersion and radiation polymerization tecnique, in which the effect of radiation polymerization on the property of the particles was studied. The relationship between enzymatic activity and irradiation temperature is shown in Fig.8. The enzymatic activity of immobilized enzyme particles obtained by irradiation at low temperatures was relatively high but the enzymatic activity decreased with increasing irradiation temperature. The decrease in the enzymatic activity at temperature above 0 °C is mainly due to the deactivation of the enzyme by irradiation. The variation of the enzymatic activity of immobilized enzyme particles with temperature was similar to that

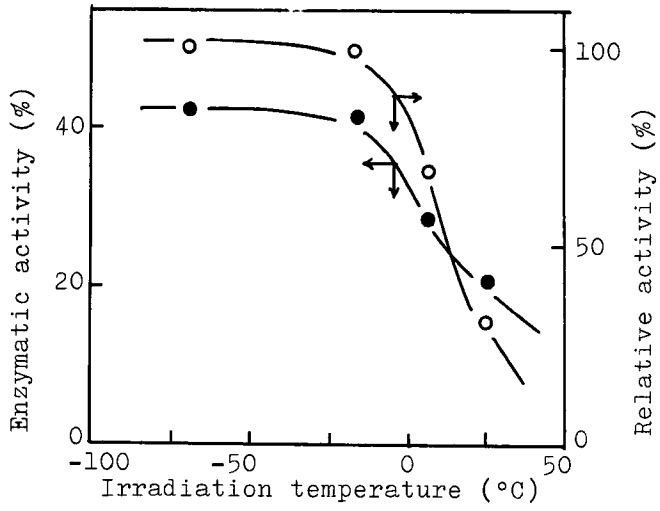
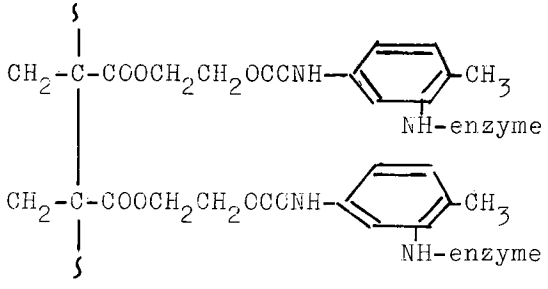


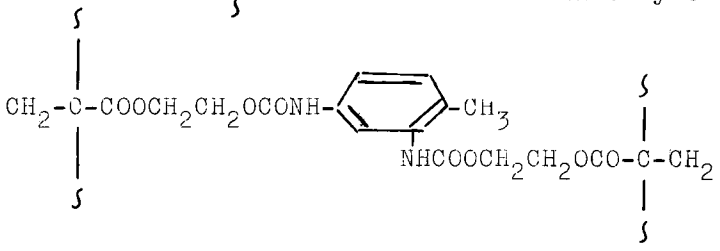
Fig.8 Effect of irradiation temperature on native enzyme (o) and immobilized enzyme particles (●). TDI/HEMA=1/1; monomer concentration, 10%(V/V)

of the relative activity of native enzymes. From investigations concerning the irradiation of native enzyme, it was found that the activity of the enzyme was unchanged by irradiation at temperatures below 0°C. The HEMA used was a glass-forming monomer at low temperatures and it can easily be polymerized by irradiation under these conditions.[12] Thus, radiation polymerization at low temperatures using HEMA was very convenient. Furthermore, using the present method, the coagulation of the dispersed particles during irradiation was avoided as the system was frozen at low temperatures.

The structure of the polymer in immobilized enzyme particles seemed to be very complex, owing to the presence of various polyurethane prepolymers. The following is proposed as the structure of the polymer chains:



(1)



(2)

The polymer chains which contribute to the binding of the enzyme are of type (1). Such polymer chains would be found mostly on the surface of the particles, because the particles are formed simultaneously with the binding of the enzyme in the dispersion step. Since the agitation of the polyurethane prepolymer in the aqueous solution is carried out rapidly, it is thought that polymer chains having non-bound isocyanate groups exist primarily within the particles.

6. Effect of particle diameter on enzymatic activity

The enzymatic activity varied with the diameter of the particles as shown in Fig.9, in which the same amount(weight) of immobilized enzyme particles with various particle sizes was used. The enzymatic activity decreased with increasing particle diameter. The surface of the immobilized enzyme particles had a slightly uneven structure. Such a structure further increases the surface area of the particles, so that a larger amount of the enzyme is immobilized.

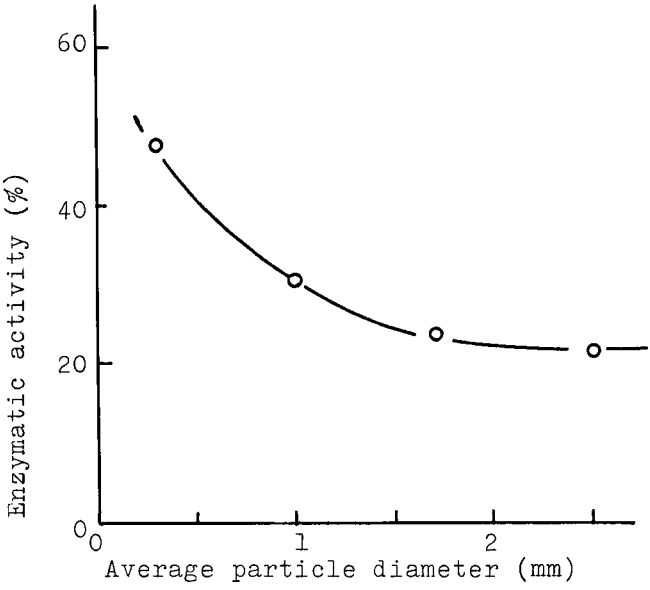


Fig.9 Effect of particle diameter on enzymatic activity. TDI/HEMA= 1/1

From these results, it was found that immobilized enzyme particles which have various particle sizes may be obtained in one step which includes dispersion and immobilization with radiation polymerization of a polyurethane prepolymer, and their properties can be varied with the conditions of dispersion and irradiation. The particles obtained by the combination of dispersion and radiation polymerization technique were of considerable rigidty. The present method could be used for the immobilization of various biological substances.

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