280. Immobilized Enzyme Particles Prepared by Radiation Polymerization of Polyurethane Prepolymer

by **Minoru Kumakura*** and **Isao Kaetsu**

Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Takasaki, Gunma, Japan

(4.X.83)

Summary

The immobilization of enzymes such as cellulase by radiation polymerization of dispersed polyurethane prepolymer was studied using tolylene-2,4-diisocyanate and 2-hydroxyethyl methacrylate. The polyurethane particles were obtained by the dispersion of polyurethane prepolymer followed by radiation polymerization, in which the enzyme was immobilized on its surface by covalent bonding. The particle diameter of immobilized enzyme particles varied with monomer concentration and composition. The enzymatic activity of immobilized enzyme particles varied with the temperature of dispersion and irradiation, and decreased with increasing particle diameter.

Introduction. – The study of immobilized enzymes as well as immobilized cells has been the subject of increased interest. The enormous efforts in this field have indicated that no immobilization technique can be expected to have universal applicability. In general, for each enzyme and each application it will be necessary to adopt a different method of immobilization. When an enzyme is trapped inside or covalently bonded to a polymer matrix, it will be distributed throughout the network of the polymer. In reacting with such immobilized enzymes, the substrate will exhibit a range of diffusion times while progressing from the liquid phase to the bound enzyme. Similarly, the products of the enzyme reaction will also show a range of diffusion times in progressing from the bound enzyme back into the external liquid phase. Thus, to reduce the diffusion time of both substrate and product to a minimum, the method of immobilizing the enzyme or the surface of a water-insoluble particle or film which has a large surface area has been tried using glass beads, [1] [2] stylene, [3] acrylamide, [4] and acrylic monomers [5]. Many of the hitherto developed methods of preparing immobilized enzyme particles involved many steps for the formation of particles and the binding of the enzyme.

In this work, a new method is described for the preparation of immobilized enzyme particles, in which the formation of the particles and the binding of the enzyme were performed in one step by radiation polymerization of a dispersed polyurethane prepolymer.

Experimental. – *Materials.* Tolyl 2,4-diisocyanate (TDI) was obtained from *Wako Pure Chemical Industries, Ltd.* and 2-hydroxyethyl methacrylate(HEMA) from *Mitsubishi Gas Chemical Co., Ltd.* The cellulase was obtained from *Yakult Mfg. Co., Ltd.*

Preparation of Immobilized Enzyme Particles. TDI and HEMA monomers were mixed, and allowed to react for 1–5 min, after which 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 $-78°$. The γ-ray irradiation (1×10^6 rad) of the flask was carried out at $-78°$ for 1 h. After irradiation, immobilized enzyme particles were obtained by warning the mixture to r.t.

Enzymatic Activity of Immobilized Enzyme Particles. The enzymatic activity (%) was obtained from the glucose formation ratio of the immobilized and native enzymes in the reaction (1.0 h at 40°, pH 4.5) of each batch of enzyme using 1.0% methoxycarbonylcellulose sodium(CMC) solution. The glucose was measured with glucose specific reagent, 'GOD-PODLK', obtained from *Nagase Sangyo Co., Ltd.*

Results and Discussion. – *Effect of Dispersion Conditions on Particle Diameter.* The effect of dispersion conditions on particle size and the stability of the particles was studied. The effect of monomer(TDI and HEMA) concentration on particle diameter is shown in *Fig. 1.* The particle diameter increased with increasing monomer concentration. At

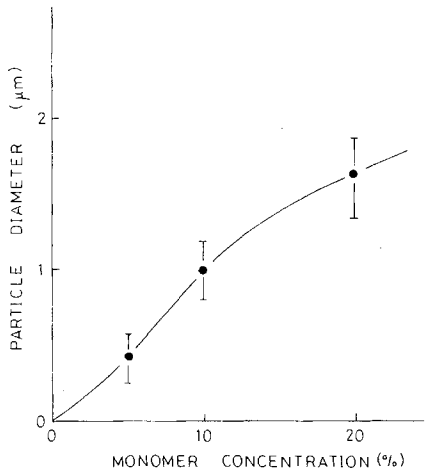
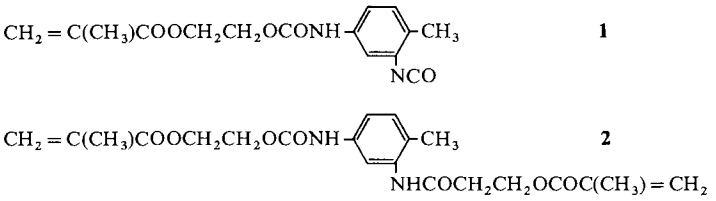


Fig. 1. *Effect of monomer concentration on particle diameter.* Monomer: TDI and HEMA; monomer composition: 1 : 1 (mol/mol).

monomer concentrations above *ca.* 30%, the formation of the particles was not observed. In the present method, the enzyme acts as a dispersion agent stabilizing the particles of polyurethane prepolymer. The presence of enzyme in the dispersion step of the preparation process was very important, because the particles were formed due to the binding of the enzyme to the prepolymer. The formation mechanism of immobilized enzyme particles is considered to be as follows. TDI reacts partially with HEMA (which has a hydroxy group) giving a polyurethane prepolymer, in which most of the molecules still contain an unreacted isocyanate group. The polyurethane prepolymer could consist of the compounds **1** and **2**.



1

2

The polyurethane prepolymer is dispersed in the aqueous solution containing the enzyme by stirring and binds the enzyme, forming particles. The particles are polymerized by irradiation at low temperatures, and become rigid. Thus, the polyurethane prepolymer appears to be dispersed by covalent binding to the enzyme. In dispersion agents such as polyoxyethylene sorbitate monostearate and sodium dodecyl sulfate instead of enzyme, particles were not formed because the agents could not bind covalently to the polyurethane prepolymer. The polyurethane prepolymer is viscous with hydrophobic properties, and molecular cohesion energy is very large, 8.74 kcal/mol [6]. In general, it is known that the order of reactivity of the isocyanate group with other compounds is as follows: $RNH_2 > R_2NH > RCH_2OH > H_2O > RCOOH$ [7]. Thus, the polyurethane prepolymer had a higher reactivity towards enzymes having amino groups than towards other compounds such as water in the aqueous solution.

The particle size varied with the composition of the monomer as well as the monomer concentration as shown in *Fig. 2*. The particle diameter had a minimum when the ratio of monomers was 1 : 1 and increased with an increasing excess of TDI or HEMA. This result indicates that one isocyanate group of TDI reacts equivalently with HEMA and the other isocyanate group of TDI reacts with the enzyme, thus forming particles. Increasing the relative concentration of TDI or HEMA results in the formation of a bulk form. Thus, the optimum composition for the formation of the particles appeared to be a ratio of 1 : 1, and this would not vary with the monomer and enzyme concentration.

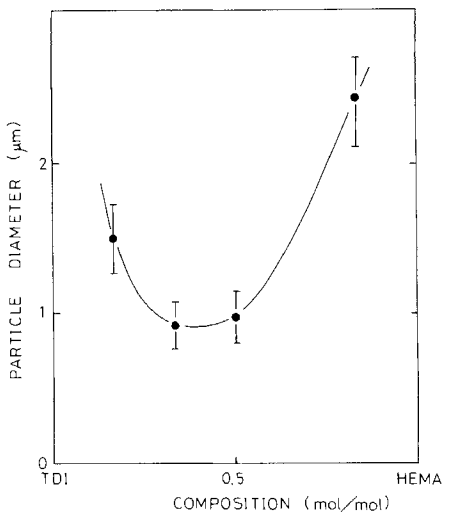


Fig. 2. *Effect of monomer composition on particle diameter.* Monomer concentration: 10% (v/v).

The particle size varied slightly with enzyme concentration though the data are not presented here. At a certain monomer concentration (10%), the critical enzyme concentration for obtaining particle appeared to be *ca.* 0.1%. At enzyme concentrations below this value, the particles were unstable and gave rise to a bulk form, while, at enzyme concentrations as high as 3%, the particle size did not decrease markedly, the particle diameter being 0.5–1 mm. The light microphotograph of immobilized enzyme particles is shown in *Fig. 3.*

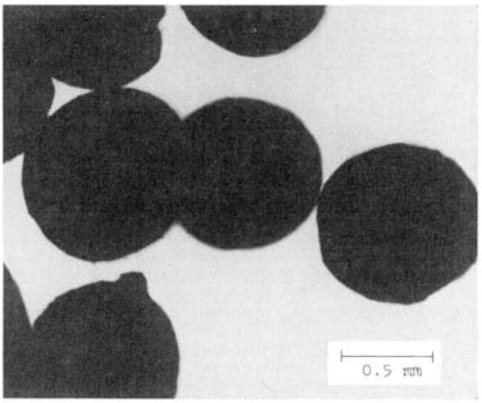


Fig. 3. *Optical microphotograph of immobilized enzyme particles.* Monomer composition: 1 : 1 (mol/mol); monomer concentration; 10% (v/v).

Effect of Dispersion Temperature on Enzymatic Activity. The effect of the dispersion conditions on the enzymatic activity of immobilized enzyme particles was studied. The enzymatic activity of immobilized enzyme particles which were obtained at various dispersion temperatures was examined, and the result is shown in *Fig. 4.* The enzymatic activity decreased slightly with increasing dispersion temperature, indicating that the enzyme is slightly deactivated by TDI at increased temperatures. This deactivation would

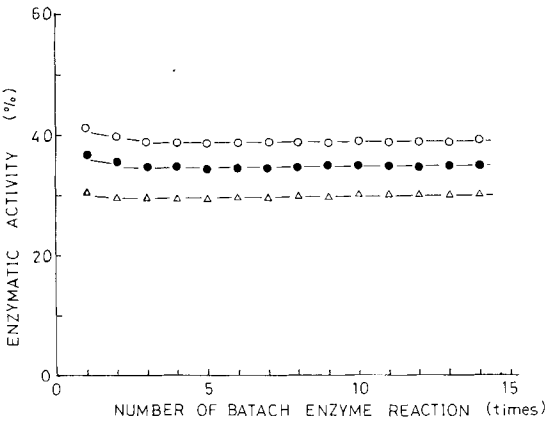


Fig. 4. *Effect of dispersion temperature on enzymatic activity.* Monomer composition: 1 : 1 (mol/mol); monomer concentration: 10% (v/v); dispersion temperature; ○ 0°, ● 10°, △ 30°.

be decreased by a decrease in the time of the dispersion operation. The enzymatic activity did not vary with the number of batch enzyme reaction and this indicated that leakage of the enzyme from the particles did not occur.

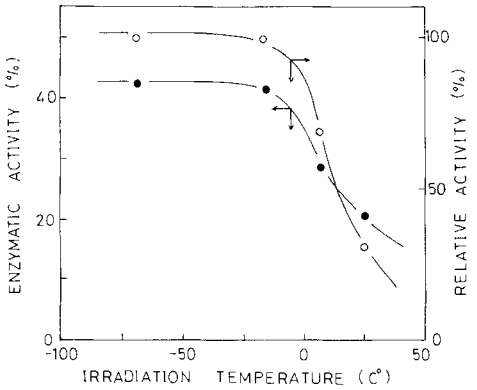
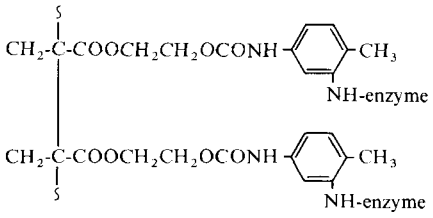


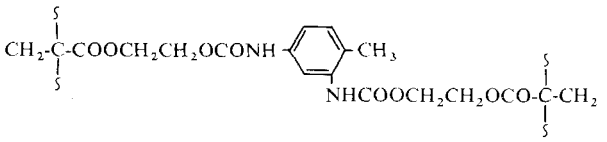
Fig. 5. *Effect of irradiation temperature on native enzyme (○) and immobilized enzyme particles (●).* Monomer composition: 1:1 (mol/mol); monomer concentration; 10% (v/v); dispersion temperature: 10°.

Effect of Irradiation Temperature on Enzyme. The relationship between enzymatic activity and irradiation temperature is shown in *Fig. 5*. 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 enzymatic activity at temperatures above 0° is mainly due to the deactivation of the enzyme by irradiation. The variation of the enzymatic activity of immobilized enzyme particles with irradiation temperature was similar to that 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°. The HEMA used was a glass-forming monomer at low temperatures and it can easily be polymerized by irradiation under these conditions [8]. 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 are proposed as structure of the polymer chains.



3



4

The polymer chains which contribute to the binding of the enzyme are of type **3**. 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.

Effect of Particle Diameter on Enzymatic Activity. The enzymatic activity varied with the diameter of the particles as shown in *Fig. 6*, 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 as can be seen in *Fig. 3*. Such a structure further increases the surface area of the particles, so that a larger amount of the enzyme is immobilized.

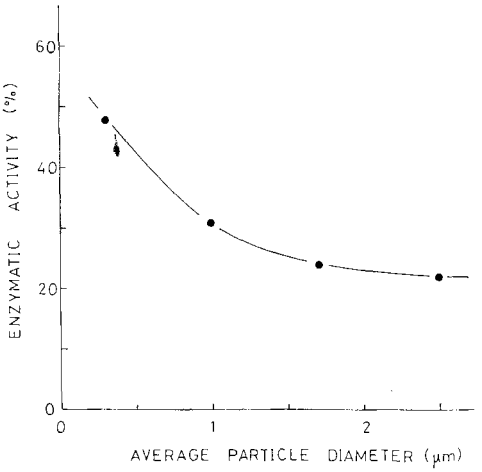


Fig. 6. *Effect of particle diameter on enzymatic activity.* Monomer composition: 1:1 (mol/mol); dispersion temperature: 10°.

Conclusion. – 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 present method could be used for the immobilization of various biological substances.

REFERENCES

[1] *R.O. Mason & H.H. Weetal*, Biotechnol. Bioeng. *16*, 637 (1972).
[2] *A.R. Neurath & H.H. Weetal*, FEBS Lett. *8*, 253 (1970).
[3] *H. Fillipusson & W.E. Hornby*, Biochem. J. *120*, 215 (1970).
[4] *P.D. Weston & S. Avvameas*, Biochem. Biophys. Res. Commun. *45*, 1574 (1971).
[5] *A. Johanson, J. Lundberg, B. Mattiasson & K. Mosbach*, Biochem. Biophys. Acta *304*, 217 (1976).
[6] *C.W. Bunn*, J. Polym. Sci. *16*, 323 (1955).
[7] *Y. Iwakura*, in 'Kindaikogyokagaku', Vol. 17, eds. R. Oda, S. Makishima, M. Imoto, W. Sakai and Y. Iwakura, Asakura Shoten, Tokyo, 1966, p. 425.
[8] *I. Kaetsu, H. Okubo, A. Ito & K. Hayashi*, J. Polym. Sci., A-1 *10*, 2203 (1972).

Erratum

Helv. Chim. Acta *66*, 2362 (1983), No. 234, by *Gamal Mikhail* and *Martin Demuth:*

p. 2364: The formula for compounds **4a** and **4b** in the *Table* has been printed as mirror image and represents therefore the wrong absolute configuration for **4b**.

Correct formula: