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Immobilization of Microbial Cells and Enzymes With Hydrophobic Photo-Crosslinkable Resin Prepolymers

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Summary. Attempts were made to entrap enzymes or microbial cells with water-insoluble photo-crosslinkable resin prepolymers of different types in organic solvent systems in the presence or absence of water. Acetone-dried cells of *Arthrobacter simplex* immobilized in a maleic polybutadiene gel (PBM-2000) converted hydrocortisone to prednisolone in a phosphate buffer. 4-Androstene-3,17-dione was converted to androst-1,4-diene-3,17-dione in benzene-*n*-heptane solution by *Nocardia rhodocrous* which was immobilized by a hydrophobic prepolymer, ENTP-2000. The ENTP-2000 had been synthesized from poly(propylene glycol)-2000, hydroxyethylacrylate and isophorone diisocyanate. Even enzymes catalyzing aqueous phase reactions, such as catalase and invertase, were immobilized in a polybutadiene resin (PB-200k) to give active gel-entrapped preparations. The cells and enzymes immobilized in these hydrophobic resins exhibited moderate activities compared with those of the free cells and enzymes.

Although various methods have been proposed with respect to the entrapment of microbial cells and enzymes, much attention is being devoted to the search for new materials and novel techniques for the formation of gels having specific characteristics. For instance, we reported convenient methods to entrap enzymes (Fukui et al., 1976; Fukushima et al., 1978; Tanaka et al., 1977a and 1978a) yeast microbodies (Tanaka et al., 1977b and 1978b), and microbial cells (Kimura et al., 1978) with photo-crosslinkable resin prepolymers and hydrophilic urethane prepolymers.

In the case of the transformation of highly hydrophobic substrates, such as steroids, terpenoids, and various organic compounds, with immobilized microbial cells or enzymes, the use of organic solvents combined with the highly lipophilic character of gel-entrapped biocatalysts would be very effective for the reaction system. The solubility of the substrates as well as the products would be significantly enhanced and the hydrophobicity of the gels would make their diffusion through gel matrices much easier. The entrapping methods reported hitherto, however, employ only hydrophilic polymers, prepolymers, or monomers as the starting material for gel formation. Therefore, we

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Abbreviations used: 4-AD, 4-androstene-3,17-dione; ADD, androst-1,4-diene-3,17-dione

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tried to immobilize microbial cells and enzymes with resin prepolymers of high hydrophobicity. Various kinds of hydrophobic prepolymers which can be crosslinked under mild conditions are available from commercial sources. Although the use of hydrophobic monomers and polymers for the microencapsulation of biological materials has been reported (Chang, 1964; Kitajima and Kondo, 1971), these methods seem to be rather complex.

This paper deals with the successful immobilization of enzymes and microbial cells inside gel matrices formed by short-time illumination of hydrophobic photo-crosslinkable prepolymers under mild conditions. Some properties of the microbial cells and enzymes thus immobilized are also described.

Materials and Methods

Photo-Crosslinkable Prepolymers

(1) Polybutadiene, UBEPOL-100 (mol wt = 200,000, abbreviated as PB-200k), was obtained from Ube Industries Ltd., Japan. (2) Maleic polybutadiene (Ube Industries Ltd., abbreviated as PBM-2000) was prepared by the following method: 100 g of maleic polybutadiene (mol wt = 2000) was esterified with 30 g of 2-hydroxyethylmethacrylate. The obtained prepolymer (130 g) was further esterified with 30 g of di(ethylene glycol) monomethacrylate and then 1.5 g of benzoin ethyl ether was added as a sensitizer. (3) A derivative of poly(propylene glycol)-2000 (abbreviated as ENTP-2000), synthesized by a similar method to the ENT prepolymer (Tanaka et al., 1978b), was obtained from Kansai Paint Co., Ltd., Japan.

Organisms

Arthrobacter simplex ATCC 6946 was grown at 30°C for 32 h with shaking (220 rpm) in a medium composed of 10 g of meat extract, 10 g of bacto-peptone, 5 g of glucose, 8 ml of 1 N NaOH, 5 g of NaCl and 1 l of tap water, followed by the induction of steroid Δ^1-dehydrogenase for 5-6 h by adding hydrocortisone (1 mg/ml) to the medium. *Nocardia rhodocrous* NCIB 10554 was grown similarly in a medium composed of 10 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 10 mg of $CaCl_2.2H_2O$, 10mg of $FeSO_4.7H_2O$, 100 mg of $MgSO_4.7H_2O$, 10 g of glycerol and 1 l of tap water. Induction of steroid Δ^1-dehydrogenase was carried out for 10 h by adding 4-AD (1 mg/ml) to the medium after 6 h of cultivation.

Enzymes and Chemicals

Yeast invertase, hydrocortisone, prednisolone and ADD were obtained from Sigma, USA. Bacterial catalase was obtained from Nagase Ind. Co., Japan, and 4-AD and Tween 85 from Nakarai Chemicals Ltd., Japan.

Analytical Methods

The amounts of hydrocortisone and prednisolone were measured as follows: A 0.5 ml of reaction solution was added to 0.5 ml of methanol dissolving a known amount of diphenylsulfoxide as authentic standard. The mixture was developed twice on a thin-layer plate (Silica gel 60 F-254, Merck, Germany) with a mixture of benzene-acetone (1:1, vol/vol). The R_f values of hydrocortisone and prednisolone with the above

mentioned solvent system were 0.56 and 0.53 respectively. The amounts of these steroids on the plate were estimated from absorption at 246 nm using a Shimadzu TLC scanner CS-910. The amounts of 4-AD and ADD were assayed by gas chromatography with the JEOL JCG-20 KFL gas chromatograph equipped with a hydrogen flame ionization detector. A glass column, 1 m x 3 mm (inner diameter), was packed with 2% Silicone AN-600 on Chromosorb WAW DMCS, 80/100 mesh (Gasukuro Kogyo Co., Ltd., Japan). The temperature of the injector and detector was 250°C. The column was maintained at 230°C with a helium gas flow rate of 40 ml/min. Under these conditions, the retention time of 4-AD and ADD was 5.30 min and 7.20 min respectively. Peak area was determined by triangulation and dehydro-*iso*-androsterone was used as an internal standard. Glucose liberated from sucrose was assayed by the coupled reaction with GODLK (glucose oxidase-peroxidase-chromogen reagent, Nagase Ind. Co., Japan). Catalase activity was measured by the electrode method described previously (Tanaka et al., 1978a). Protein was estimated by the method of Lowry et al. (1951).

Results

Immobilization of Arthrobacter Simplex Cells With PBM-2000

Two-hundred milligram of *A. simplex* acetone-dried cells were suspended in 2.0 ml of water-acetone mixture (1:19, vol/vol) containing 1.0 g of PBM-2000 prepolymer. The mixture was layered on a sheet of transparent polyester, and radiated from appropriately placed UV light source for 3 min as described previously (Fukui et al., 1976;

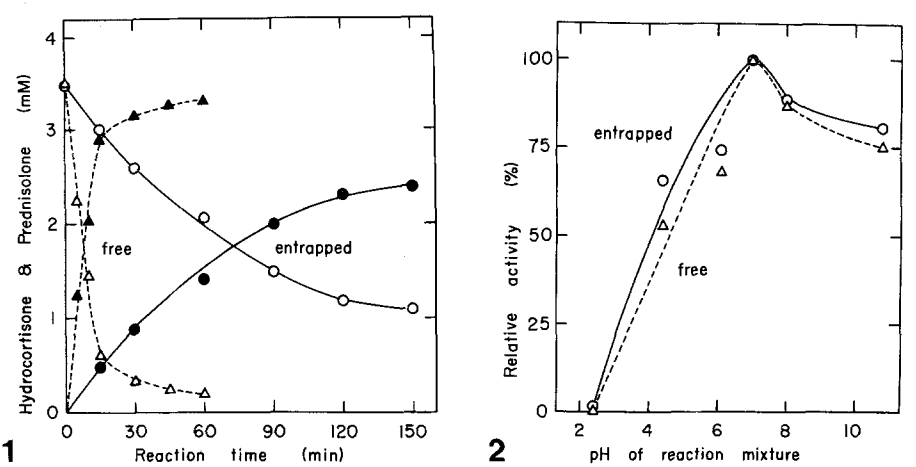
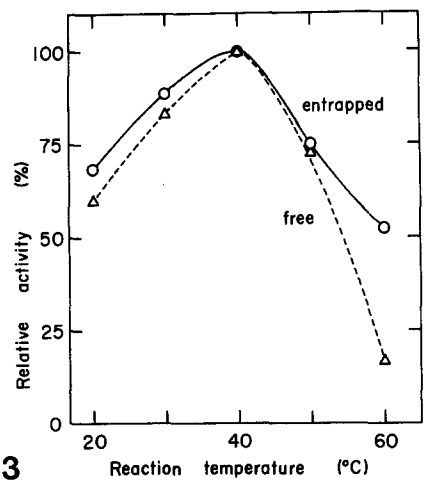


Fig. 1. Time-course of transformation of hydrocortisone to prednisolone by free and gel-entrapped acetone-dried cells of *Arthrobacter simplex*. (○, △), Hydrocortisone; (●, ▲), prednisolone: (○, ●), PBM-2000-entrapped cells; (△, ▲), free cells

Fig. 2. pH-dependency of hydrocortisone transformation by free and gel-entrapped acetone-dried cells of *Arthrobacter simplex*. The activities of free (△) and PBM-2000-entrapped cells (○) were assayed at indicated pH



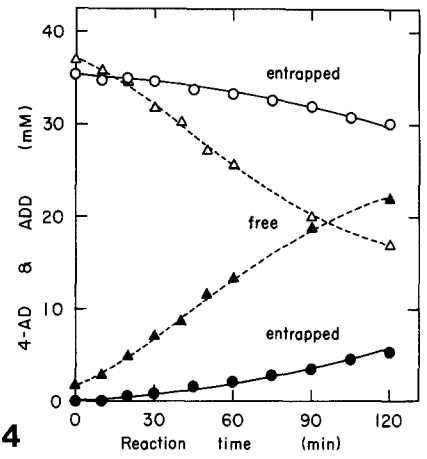


Fig. 3. Temperature-dependency of hydrocortisone transformation by free and gel-entrapped acetone-dried cells of *Arthrobacter simplex*. The activities of free (△) and PBM-2000-entrapped cells (○) were assayed at indicated temperature

Fig. 4. Time-course of transformation of 4-AD to ADD by free and gel-entrapped *Nocardia rhodocrous* cells. (○, △), 4-AD; (●, ▲), ADD: (○, ●), ENTP-2000-entrapped cells; (△, ▲), free cells

Tanaka et al., 1977a and 1978b). A resin film thus formed was cut into small pieces (each approx. 5 x 5 mm) and used for transforming hydrocortisone. 20 mg of hydrocortisone and 200 mg of the immobilized *A. simplex* cells or free acetone-dried cells were suspended in 20 ml of 20 mM potassium phosphate buffer (pH 7.0). The conversion of hydrocortisone to prednisolone was carried out at 30°C with shaking (120 strokes/min). Figure 1 illustrates a typical time-course change as hydrocortisone is consumed and prednisolone formed. The rate of the transformation of hydrocortisone to prednisolone with the immobilized cells was 192 nmol h^{-1} mg dry $cell^{-1}$, while that of the free cells was 1104 nmol h^{-1} mg dry $cell^{-1}$.

Figure 2 shows the pH-dependency of the steroid transformation reaction, indicating no appreciable difference between the immobilized and free cells. The temperature dependency of the steroid transformation reaction is illustrated in Figure 3. The entrapped cells showed a slightly higher relative activity than the free cells at the same temperature. Thus, the acetone-dried cells of *A. simplex* entrapped in the highly hydrophobic gel showed a moderate activity in converting hydrocortisone to prednisolone in an aqueous phase. The steroid transformation in organic solvents or water-organic solvent systems, however, did not proceed under the conditions employed. The cells entrapped with PB-200k also showed a similar behavior.

Immobilization of Nocardia rhodocrous With ENTP-2000

One gram of *N. rhodocrous* wet cells, suspended in 2.0 ml of benzene and sonicated for 30 s, were mixed with 1.0 ml of benzene containing 1.0 g of ENTP-2000 prepolymer and 10 mg of benzoin ethyl ether as an initiator. The cell-prepolymer mixture

was photo-crosslinked as described above. The resin film entrapping *N. rhodocrous* cells was cut into small pieces (each approx. 1 x 1 mm) and used for the transformation of 4-AD. As shown in Figure 4, the immobilized cells as well as the free cells converted 4-AD to ADD in a water-saturated mixture of benzene and *n*-heptane (1:1, vol/vol) in the presence of phenazine methosulfate (1.63 mM), which acted as an artificial electron acceptor. The maximal rate of the transformation of the immobilized cells was about 23% of that of the free cell.

The cells entrapped with PBM-2000 did not show any transformation activity. The gel formed with PB-200k could not be used in this system because the gel would become significantly swollen in the benzene and *n*-heptane mixture.

Immobilization of Invertase With PB-200k

Invertase dissolved in 0.1 ml of 20 mM acetate buffer (pH 4.9; protein, 66 μg) and 0.05 ml of Tween 85 were mxied thoroughly with 10 ml of benzene containing 1.0 g of PB-200k, 10 mg of benzoin ethyl ether and 30 mg of benzoylperoxide. The mixture was then illuminated as described above. The amount of Tween 85 added was changed from 0.05 ml to 0.3 ml. The resin film thus formed was cut into small pieces (each approximately 5 x 5 mm) and used as a test sample of immobilized invertase. The apparent K_m value of the immobilized enzyme (133 mM) was about twice of that of the free enzyme (77 mM). The V_{max} value of the immobilized enzyme (0.29 mmol min^{-1} mg $protein^{-1}$) was one-third of that of the free enzyme (0.81 mmol min^{-1} mg $protein^{-1}$).

No appreciable difference was observed between the pH-activity profiles of immobilized and free invertase (Fig. 6). The temperature dependency of the invertase reaction is shown in Figure 7. The optimal temperature for the free enzyme was 55°C, while that for the immobilized enzyme was 50°C. The stability of the immobilized enzyme over repeated reactions was also examined. As illustrated in Figure 8, the enzyme was stable through 20 batch reactions (total operational period, 10 h) without any appreciable loss of the activity both in the 25 mM sucrose and in the 50 mM sucrose.

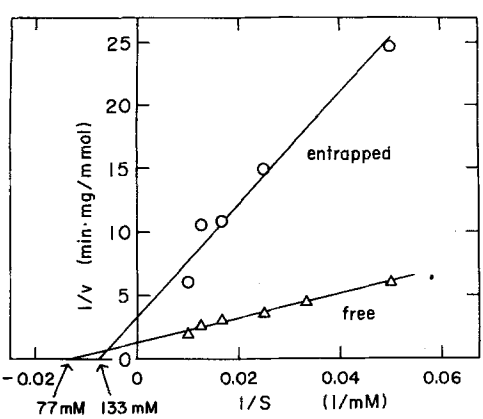
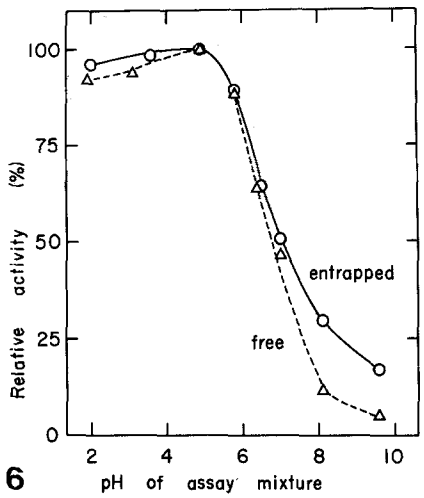


Fig. 5. Lineweaver-Burk plots of free and PB-200k-entrapped invertase. Reaction was carried out at 30°C. (△), Free invertase; (○), entrapped invertase



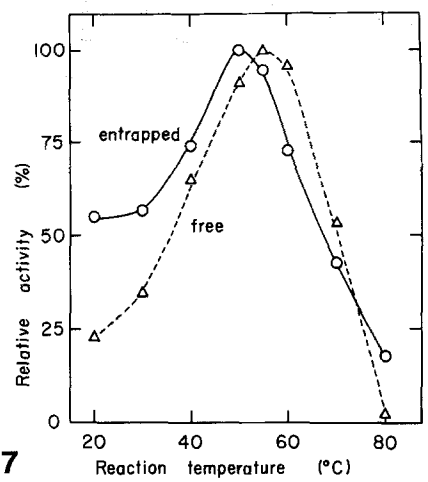


Fig. 6. pH-dependency of invertase reaction. The activities of free (△) and PB-200k-entrapped invertase (○) were assayed at indicated pH. Reaction was carried out at 30°C

Fig. 7. Temperature-dependency of invertase reaction. The activities of free (△) and PB-200k-entrapped invertase (○) were assayed at indicated temperature. Reaction was carried out at pH 4.9

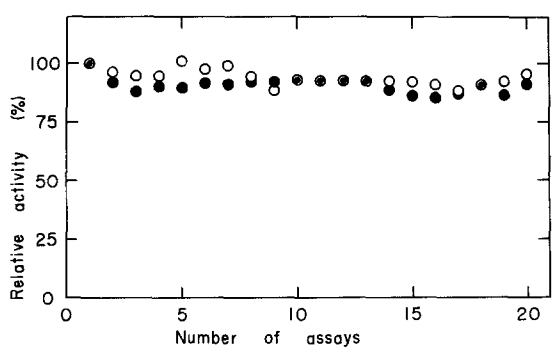


Fig. 8. Repeated use of PB-200k-entrapped invertase. Each assay was carried out at 30°C for 30 min with 25 mM (○) or 50 mM (●) sucrose

The activity of invertase entrapped with PBM-2000 or ENTP-2000 was lower than that of the enzyme entrapped with PB-200k.

Immobilization of Catalase With PB-200k

42.7 mg of catalase obtained from a commercial source was dissolved in 10 ml of 20 mM potassium phosphate buffer (pH 7.0) and the solution was centrifuged at 2,000 x g for 10 min to obtain a clear supernatant. 0.7 ml of the supernatant solution (protein, 175 μg) and 0.3 ml of Tween 85 were mixed thoroughly with 10 ml of benzene containing 1.0 g of PB-200k, 10 mg of benzoin ethyl ether, and 30 mg of benzoylperoxide. The PB-200k film entrapping catalase was obtained as described above, cut into small pieces (each approximately 5 x 5 mm), and used for the degradation of hydrogen peroxide.

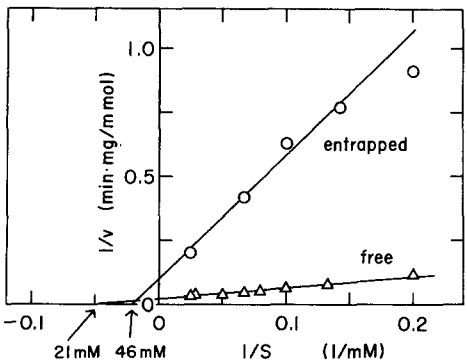
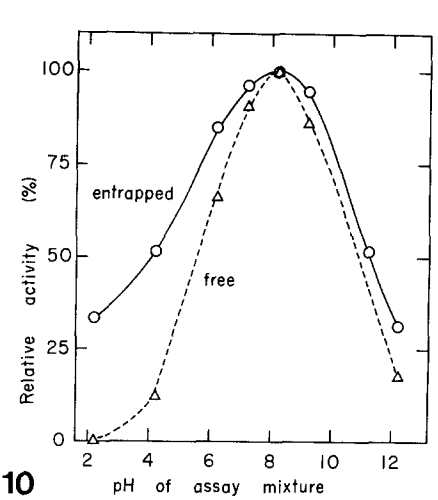


Fig. 9. Lineweaver-Burk plots of free and PB-200k-entrapped catalase. Reaction was carried out at 30°C. (△), Free catalase; (○), entrapped catalase



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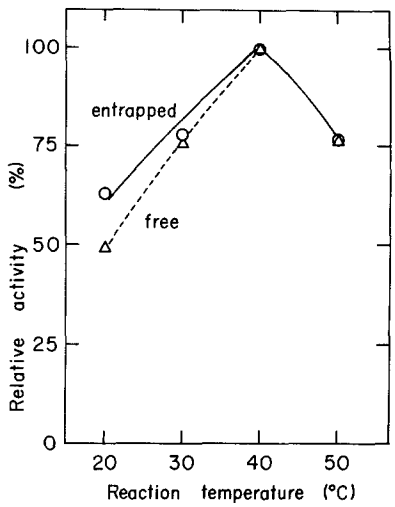

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Fig. 10. pH-dependency of catalase reaction. The activities of free (△) and PB-200k-entrapped catalase (○) were assayed at indicated pH. Reaction was carried out at 30°C

Fig. 11. Temperature-dependency of catalase reaction. The activities of free (△) and PB-200k-entrapped catalase (○) were assayed at indicated temperature

Figure 9 shows Lineweaver-Burk plots of free and immobilized catalase. The apparent K_m value of the gel-entrapped catalase (46 mM) was approximately twice of that of the free counterpart (21 mM). The V_{max} value (9.35 mmol min^{-1} mg $protein^{-1}$) was about 20% of that of the free enzyme (47.6 mmol min^{-1} mg $protein^{-1}$). A comparison of the pH-activity profiles of the free and the immobilized enzyme revealed that the latter showed a somewhat higher relative activity than the former over the pH range examined, especially at acidic pH value (Fig. 10). The temperature-activity profiles of the entrapped and free enzyme did not differ significantly (Fig. 11). Entrapped catalase was found to be relatively stable through repeated enzyme reactions as long as the concentration of the hydrogen peroxide was low, whereas a decrease in the activity was dramatical at a concentration of 20 mM (Fig. 12).

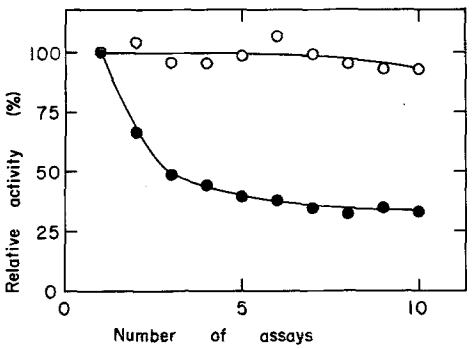

Fig. 12. Repeated use of PB-200k-entrapped catalase. Each assay was carried out at 30°C for 1 min with 1 mM (○) or 20 mM (●) hydrogen peroxide

The activity of the catalase entrapped with PBM-2000 was very low.

Discussion

This paper describes a simple and convenient method to entrap microbial cells and enzymes with photo-crosslinkable, hydrophobic resin prepolymers in organic solvent systems.

This immobilization method seems to have the following features: (1) It was easy to entrap enzymes or microbial cells in gel matrices without any loss of the protein or cells. (2) Microbial cells and enzymes entrapped in hydrophobic gels by this procedure had moderate activities even in aqueous reaction mixtures. This means that extended kinds of prepolymers, not restricted to the hydrophilic ones used so far, might be applicable for the immobilization of biological materials. (3) In the case of microbiological and enzymatic transformation of water-insoluble substrates, such as steroids, terpenoids, and various organic compounds, hydrophobic gels are considered to be superior to hydrophilic gels as far as the diffusion characteristics of these substrates are concerned. The transformation of organic compounds by immobilized cells and enzymes in organic solvents, in which the concentration of lipophilic substrates can be increased, is very attractive, since high product yields are favored. In such system, hydrophobic gel-entrapped enzymes and microbial cells are expected to be more efficient catalysts than the hydrophilic gel-entrapped ones.

The details of the transformation of steroids by the immobilized cells in organic solvents or a water-organic solvent system will be published later.

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