KINETIC STUDY OF UREA HYDROLYSIS BY UREASE IMMOBILIZED WITH BOVINE SERUM ALBUMIN AND GLUTARALDEHYDE ON POLYURETHANE FOAM

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Introduction

Enzyme immobilization and immobilized-enzyme reactors have attracted considerable attention in industrial applications.[14,19,23] Many immobilization methods have been studied.[14,22] Considering the reactor, the carrier or support of immobilized biocatalysts also plays an important role in practical application.

Polyurethane (PU) foam is not only cheap and readily available but is also flexible and has favorable hydrodynamic properties owing to its quasi-spherical membrane structure.[4] It is applicable to various reactors, such as continuous-flow column, stirred-tank reactor, squeezer, and pulsating column.[4] In 1965, Bauman *et al.* first immobilized enzyme-containing starch gel physically on open-cell PU foam to prepare an enzyme pad for analytical uses.[1] Because PU is usable in aqueous solution and nonaqueous or microaqueous organic solvent systems, the immobilization of biocatalysts in PU gel or PU foam has received much more attention since 1978.[8-11,15,21] In addition, open-cell PU foam has also been used as the carrier for cell culture in various bioreactors due to its chemical inertness, mechanical stability, and porous property.[16,18] In this work, the immobilization of urease on the surface of PU foam with bovine serum albumin and glutaraldehyde was studied.

1. Experimental

1.1 Materials

Urease from jack beans (Type III) was purchased from Sigma Chemical Co. (St. Louis, Missouri). Urea and bovine serum albumin were guaranteed reagents of E. Merck (Darmstadt). Glutaraldehyde (50% in water) was obtained from Fluka Chemie AG, Switzerland. Except those used for preparing PU foam, all chemicals were E. P. grade or commercially available guaranteed reagents.

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The PU foam, prepared by a one-shot method,[20] had a bulk density of about $30\,kg\,m^{-3}$ and a porosity of about 0.96 (v/v). Prior to the immobilization of urease, PU foam was washed with water and cut into thin sheets of $2 \times 1.5\,cm^2$ with a thickness of 1 mm.

1.2 Immobilization of urease on PU foam

A typical film-preparing enzyme solution was prepared by dissolving 0.6 g bovine serum albumin and 0.02 g urease in $10\,cm^3$ of $0.02\,mol\,dm^{-3}$ phosphate buffer containing 0.25% glutaraldehyde at pH 7.0 and 4°C.[3] Unless otherwise specified, the typical solution was used for the immobilization of urease in this work. Urease was immobilized according to the following procedure. PU foam was first immersed in the film-preparing enzyme solution and squeezed by a glass rod to remove the air trapped in the foam. The PU foam was then removed and brushed gently on the upper rim of the container to remove excess solution. Finally it was air-dried at 25°C. After drying (about 10 hours), the immobilized enzyme PU foam was washed with $0.02\,mol\,dm^{-3}$, pH 7.0, cool phosphate buffer until the washings were free of glutaraldehyde and noncrosslinked urease and bovine serum albumin.

The dried immobilized enzyme PU foam was slightly rigid, responding to the character of the enzyme film supported. However, the flexibility of PU foam was recovered in aqueous solution because the enzyme film was hydrophilic. Furthermore, the favorable hydrodynamic property of PU foam was also retained because the supported enzyme film was rather thin relative to the apparent pore size of PU foam.

Since PU foam was an inert support, the weight of urease immobilized was estimated to be: (weight of enzyme film) × (weight percentage of urease relative to the sum of urease and bovine serum albumin in film-preparing enzyme solution).

1.3 Measurement

To make PU foam and reaction solution contact well, immobilized enzyme PU foam was first impregnated with water and then squeezed and dried

by a filter paper to remove excess water. After being cut into fine pieces, the foam was dropped into 2 cm^3 of 0.1 mol dm^{-3} urea-containing phosphate buffer solution to initiate the reaction. The reaction solution was kept at the desired temperature and stirred vigorously with a magnetic stirrer to minimize aqueous film resistance. At each preset time interval, 0.1 cm^3 of reaction solution was pipetted out for analysis of ammonium ion concentration.[6]

The activity of immobilized urease was determined by the least squares method using three sampling points taken within an early 3-minutes period in each run. The specific activity of immobilized urease was calculated on the basis of the estimated amount of urease immobilized. Unless otherwise stated, the activity of immobilized urease was measured at pH 7.0, 25°C, and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer. All properties of free urease were obtained from our previous study.[12]

2. Results and Discussion

2.1 Effects of the concentrations of glutaraldehyde and film-preparing solution on the immobilization of urease

To investigate the effects of the concentrations of glutaraldehyde and film-preparing solution on the immobilization of urease, the original film-preparing enzyme solutions used were similar to the typical one described in section 1.2, but with glutaraldehyde concentrations ranging from 0.05 to 3.0%. The diluted film-preparing enzyme solutions were prepared by further diluting the above solutions to the desired dilution ratios 1.3, 2 and 3, using 0.02 mol dm^{-3} phosphate buffer of pH 7.0.

The crosslinking percentages of the enzyme film from original film-preparing enzyme solutions (dilution ratio 1) were 90–99% when the glutaraldehyde concentration in film-preparing enzyme solution was above 0.25%. Below this concentration, however, the crosslinking percentage decreased rapidly to zero. Furthermore, the optimum glutaraldehyde concentration in film-preparing enzyme solutions was found to be about 0.25%, as illustrated in **Fig. 1**. It also shows that the immobilized urease lost activity almost completely when glutaraldehyde concentration exceeded 1%. The loss in activity may be due to the fact that the excess glutaraldehyde reacts with SH-groups of urease molecules, which are essential for the catalytic activity of urease.[13]

Similar curves were obtained for immobilized urease prepared from the diluted film-preparing enzyme solutions, as shown in Fig. 1. The glutaraldehyde concentrations of those diluted film-preparing enzyme solutions shown in Fig. 1 were the concentrations before being diluted. Therefore, the optimum amount of glutaraldehyde in film-preparing enzyme solution was 4% relative to the total weight of bovine serum albumin and urease, independent of its real concentration in solution.

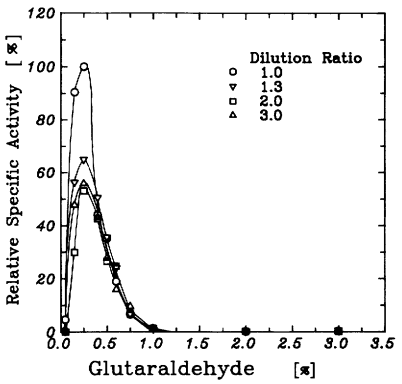

Fig. 1. Effects of concentrations of glutaraldehyde and film-preparing enzyme solution on activity of resulting immobilized urease. The activities were measured at pH 7.0, 25°C and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer

In addition, the thickness of enzyme films for the cases of dilution ratios 1, 1.3, 2, and 3 were measured to be 7–15, 5–12, 4–7, and 3–5 μm, respectively, by microscope. This indicated that the thickness of enzyme film was consistent with the concentration of total protein in film-preparing enzyme solution. However, Fig. 1 shows that the specific activity of immobilized urease did not decrease with increasing concentration of film-preparing enzyme solution. Therefore, the effect of the concentration of total protein in film-preparing enzyme solution on the specific activity of immobilized urease could be attributed to two (at least) contrary factors: the thickness of enzyme film (mass transfer effect) and the probable deactivation of urease due to dilution during immobilization.

The specific activity of immobilized urease prepared from typical film-preparing enzyme solution was measured to be 20.4 μmol NH_3 min^{-1} mg^{-1}. Compared with the specific activity of free urease obtained under the same condition (39.8 μmol NH_3 min^{-1} mg^{-1}),[12] the residual activity of urease after immobilization was about 50%.

2.2 Effect of the urease amount in film-preparing solution on activity of immobilized urease

Film-preparing enzyme solutions similar to the typical one but with the amount of urease ranging from 0.005 to 0.063 g were used for the immobilization of urease. The relationship of the activity and concentration of the immobilized urease in reaction solution, $[E]_0$, is shown in **Fig. 2**. A linear relationship was obtained when $[E]_0$ was less than 200 mg dm^{-3}, whereas the specific activity decreased beyond this amount. This could be due to a weaker protective effect of bovine serum albumin on urease activity[3] because bovine serum albumin was relatively in-

sufficient when higher urease concentration in film-preparing enzyme solution was used. Other plausible explanations might be that the real amount of crosslinked urease was less than that estimated, or that the mass transfer resistance was larger.

2.3 Effect of pH on activity of immobilized urease

The effect of pH in the range of 4.0–9.5 on the activity of immobilized urease was studied at 0.1 mol dm^{-3} urea and 25°C in 0.1 mol dm^{-3} phosphate buffer. The pH dependences of the activities of immobilized urease and free urease[12] are shown in **Fig. 3**. The optimum pH of urease was shifted from 7.2 to 7.0 after immobilization. It was also seen that immobilized urease was less sensitive to pH at pH < 7.0 and slightly more sensitive to pH at pH > 7.0 than free urease. This is in agreement with the general observation[17] and can be attributed to the change in the microenvironment of enzyme after immobilization.[5]

2.4 Effect of temperature on activity of immobilized urease

The effect of temperature in the range of 10–70°C on the activity of immobilized urease was studied at pH 7.0 and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer. **Figure 4** shows the Arrhenius plots of urea hydrolysis both by immobilized urease and by free urease,[12] in which V and T denote the specific activity of urease and the temperature respectively. For both immobilized urease and free urease, the relationships between ln V and $1/T$ remain linear up to about 40°C, and become downward beyond this temprature due to thermodenaturation of urease. Using the data in the range of 10–40°C, the activation energy for immobilized urease was calculated to be 36.4 kJ mol^{-1}, which was only slightly higher than that for free urease, 32.6 kJ mol^{-1}. This indicates that the structure of urease was not significantly changed after immobilization.

2.5 Effect of substrate concentration

The effect of substrate concentration ranging from 0.001 to 4.0 mol dm^{-3} on the activity of immobilized urease was studies in 0.1 mol dm^{-3} phosphate buffer at pH 7.0 and 25°C. The results for both immobilized urease and free urease[12] are illustrated in **Fig. 5**. For both ureases, the activities (initial rates of urea hydrolysis) followed Michaelis-Menten kinetics except at urea concentrations above 0.1 mol dm^{-3} (for immobilized urease) or 0.25 mol dm^{-3} (for free urease) where the rate decreased due to substrate inhibition. The substrate inhibition model can be shown as[7]

$$V=\frac{V_{max}}{1+K_m/S+S/K_i^s} \quad (1)$$

where V_{max} is maximum specific activity, K_m is the Michaelis constant, S is substrate concentration, and K_i^s is the substrate inhibition constant.

Using the Rosenbrock optimization method,[2] V_{max}, K_m, and K_i^s for immobilized urease were determined to be 24.9 μmol NH_3 min^{-1} mg^{-1}, 0.0198 mol dm^{-3}, and 3.95 mol dm^{-3} respectively with a mean error of 6.57%. For free urease, the values were 47.2 μmol NH_3 min^{-1} mg^{-1}, 0.0192 mol dm^{-3}, and 6.42 mol dm^{-3} respectively, with a mean error of 2.30%.[12] This

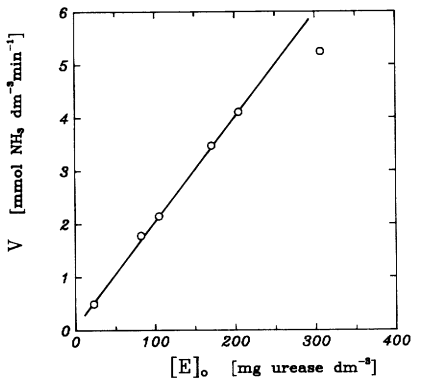

Fig. 2. Relationship of activity and concentration of immobilized urease at pH 7.0, 25°C and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer

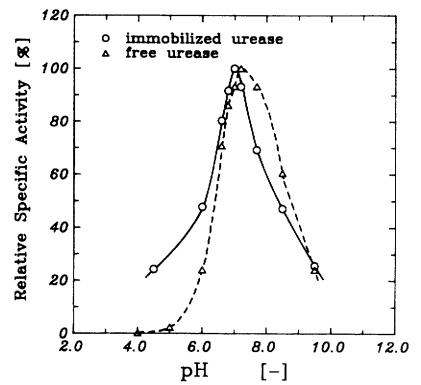

Fig. 3. Effects of pH on activity of urease at 25°C and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer

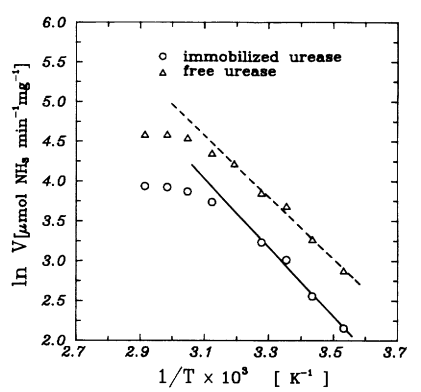

Fig. 4. Arrhenius plots of initial rates of urea hydrolysis catalyzed by urease at pH 7.0 and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer

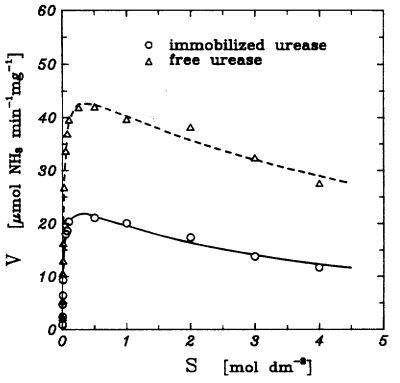

Fig. 5. Effects of substrate concentration on initial rates of urea hydrolysis catalyzed by urease in 0.1 mol dm^{-3} phosphate buffer at pH 7.0 and 25°C

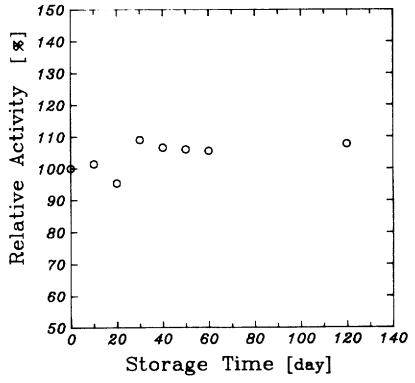

Fig. 6. Storage stability of immobilized urease in refrigerator at −10°C

indicated that the K_m value was almost unchanged, but the K_i^s value was lowered by immobilization. The curves calculated according to Eq. (1) are also shown in Fig. 5.

2.6 Stability

The specific activities of the immobilized enzymes stored in a refrigerator (about −10°C) showed no loss over the measured period of 120 days, as shown in **Fig. 6**. This indicated that the immobilized urease has excellent storage stability. At pH 7.0, 25°C and 0.1 mol dm^{-3} urea in 0.1 mol dm^{-3} phosphate buffer, it was found that the immobilized urease can be reused at least four times and without significant loss in activity. This indicated that the immobilized urease has good operation stability under the reaction condition.

Conclusion

Urease was immobilized with bovine serum albumin and glutaraldehyde on the surface of reticulated polyurethane foam. The optimum amount of glutaraldehyde was 4% relative to the weight of total protein, and was independent of its real concentration in solution. The residual activity of urease after immobilization was about 50%. The thickness of the enzyme film was less than 15 μm. The activity of urease at low pH was improved by immobilization. The activation energy of urea hydrolysis by immobilized urease is slightly higher than that by free urease. The substrate inhibition constant was lowered by immobilization. The Michaelis constant for free urease and immobilized urease were almost the same. The storage and operation stabilities were good. This study offers another simple and practicable method for enzyme immobilization, which has potential for practical applications in various bioreactors.

Acknowledgment

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Nomenclature

K_i^s	=	substrate inhibition constant	[mol dm^{-3}]
K_m	=	Michaelis constant	[mol dm^{-3}]
S	=	substrate concentration	[mol cm^{-3}]
T	=	temperature	[K]
V	=	specific activity	[μmol NH_3 min^{-1} mg^{-1}]
		or activity	[mmol NH_3 dm^{-3} min^{-1}]
V_{max}	=	maximum specific activity	[μmol NH_3 min^{-1} mg^{-1}]
$[E]_0$	=	immobilized urease concentration	[mg dm^{-3}]

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