Permeation Decomposition of Urea Through Asymmetrically Urease-Immobilized Ethylene–Vinyl Alcohol Copolymer Membranes

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ABSTRACT: Urease-immobilized ethylene–vinyl alcohol copolymer (EVA) membrane was prepared by the covalent bonding of urease on EVA membrane activated with cyanuric chloride. The urease-immobilized EVA membrane had optimum pH at about 7.0 similar to native urease. The Michaelis constant (K_m) and the maximum velocity (V_m) of the urease-immobilized EVA membrane were smaller than those of native urease. When the EVA membrane, prepared on a polyethylene (PE) plate by the phase inversion method, was used as a support, urease was asymmetrically distributed in the direction of cross section of the urease-immobilized EVA membrane. The permeation decomposition of urea through the asymmetrically urease-immobilized EVA membrane depended on the asymmetric distribution of urease in the membrane. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **63:** 1579–1588, 1997

INTRODUCTION

Over the last few years, the number of studies on enzyme immobilization has increased drastically, since immobilized enzymes have been of more interest in the boundary between biochemistry and polymer chemistry.[1–3] Unlike homogeneous catalysis, in which enzyme and substrate are present in a homogeneous solution, the immobilization of the enzyme that makes heterogeneous catalysis possible can also be a very considerable advantage. Especially, though it is generally difficult to separate efficiently the dissolved or finely suspended native enzyme from products in homogeneous catalysis, the use of an immobilized enzyme enables simple separations and continuous processes due to the heterogeneous catalysis. A membrane has a high possibility as an excellent support for enzyme immobilization, since high reaction efficiency and simple separation of products can be achieved using enzyme-immobilized membranes.[4]

Hemodialysis is performed in the treatment of patients with renal disease. An artificial kidney is mainly composed of a membrane (hemodialyzer) which separates blood from dialysate solution and supporting equipment. The blood solutes are permeated into the dialysate solution through the membrane.[5,6] Generally, cellulose, polyacrylonitrile, ethylene–vinyl alcohol copolymer (EVA), etc., are used as the membranes for the hemodialyzers. Since EVA membrane consists of a hydrophilic vinyl alcohol component and a hydrophobic ethylene component and has a good blood compatibility, its membrane is especially expected to be useful for an artificial kidney.

Conventional hemodialysis for patients with renal disease requires 100–300 L of dialysate solution per treatment. The required amount of dialysate solution can be reduced by preparing it for further use, if urea can be removed from the dialysate solution. The immobilization of urease, which is an enzyme for decomposition of urea,[7,8]

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on the membrane leads to overcoming the disadvantage of requiring the large amount of dialysate solution.

Previously we investigated the surface characteristics of EVA membranes prepared under various conditions using X-ray photoelectron spectroscopy (XPS).[9,10] The studies made it clear that the hydroxyl groups are localized at the surface of the EVA membrane. We also reported on the preparation of catalyst-immobilized EVA membranes in which the catalyst was asymmetrically distributed, and the effect of the asymmetric distribution on the permeation reaction.[11,12] The asymmetrically catalyst-immobilized EVA membranes have a possibility as high-performance reactors. Therefore, immobilization of urease on the EVA membrane may give us an excellent membrane for a hemodialysis.

This paper describes the preparation of a urease-immobilized EVA membrane and the permeation decomposition of urea through the membrane for the development of a conventional hemodialysis for patients with renal disease. In particular, urease was asymmetrically immobilized on the EVA membrane in the direction of the membrane cross section. We focused on the effect of the asymmetric distribution of immobilized urease in the membrane on the permeation decomposition of urea through the membrane.

EXPERIMENTAL

Materials

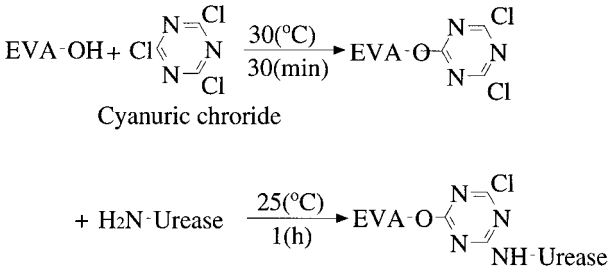
EVA having a vinyl alcohol content of 68 mol % and a degree of polymerization of 1100 (Kuraray Co., Ltd.) was used. Jack bean urease (59.7 U/mg) was obtained from Toyoboseki Co., Ltd. As the other solvents and reagents, analytical grade reagents were used without further purification. Water was distilled ion-exchanged water.

Preparation of EVA Membrane

Ten grams of EVA were dissolved in 50 mL of dimethyl acetamide (DMAc) at 80°C. EVA membrane was prepared by casting the solution on a glass or polyethylene (PE) plate and then dipping it immediately in water at 10°C (phase-inversion method).

Immobilization of Urease on the EVA Membrane

Immobilization of urease on the EVA membrane was carried out in the procedure reported by Kay and Crook[13] and Kay et al.[14] as follows (Scheme 1): The EVA membrane was immersed in successive, 1*N* aqueous NaCl solution, acetone solution of prescribed amount of cyanuric chloride, and 20% aqueous acetic acid solution at 30°C for 30 min, and then dipped in a cold aqueous acetone solution. In this procedure, the EVA membrane was activated by cyanuric chloride. The amount of cyanuric chloride in the EVA membrane, e.g., cyanuric chloride which could activate the EVA membrane, was determined by the KBr method with Fourier transform infrared spectrometer (Shimadzu Co., Ltd.: FT-IR 4200) after freeze drying of the membrane. The determination is based on the C—H stretching band at 2940 cm^{-1} and the C=N stretching band at 1500 cm^{-1}.



Scheme 1 Activation of EVA with cyanuric chloride.

The cyanuric chloride-activated EVA membrane was further immersed in an aqueous solution containing a prescribed amount of urease at 25°C, pH 7.2 for an hour. After the immobilization, the urease-immobilized EVA membrane was sufficiently washed with 2.5×10^{-2} mol/L phosphate buffer (pH 8.4) five times for removal of adsorbed urease. The amount of urease immobilized on the EVA membrane was determined with BCA protein assay reagent (Pierce).[15] The BCA protein assay reagent determines the amount of protein using the reducing reaction of the protein. The urease-immobilized EVA membrane was immersed in 2 mL of the BCA protein assay reagent at 37°C for 30 min. After the reaction, the amount of immobilized urease was obtained from the absorbance of the reagent measured with a Hitachi Model 100-50 spectrophotometer.

Measurement of Enzyme Activity

Enzyme activity was estimated by measurement of the amount of ammonia produced enzymatically, using urea as a substrate. Native urease was added or the urease-immobilized EVA membrane was immersed into 50 mL of 2.5×10^{-2} mol/

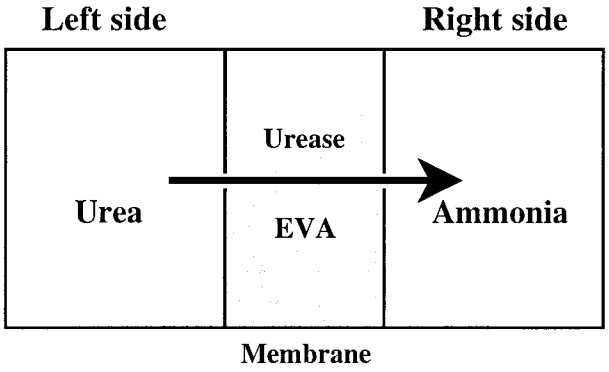


Figure 1 Schematic diagram of diaphragm cell.

L phosphate buffer containing 1.3×10^{-2} mol/L of urea, and urea was decomposed into ammonia by urease with stirring at 30°C. The amount of ammonia produced was determined spectrophotometrically by using reaction of phenol with hypochlorite by the method reported by Weatherburn.[16] Sodium nitroprusside dihydrate was added to 500 mL of water containing 5 g of phenol (Solution A). Solution B was prepared by dissolving 2.5 g of sodium hydroxide and 4.2 mL of antiformin (sodium hypochlorite solution). After 20 mL of sample was added to 5.0 mL of Solution A with sufficient stirring, 5.0 mL of Solution B was mixed thoroughly and was kept at 37°C for 20 min. The absorbance of the solution was measured at 625 nm against a reagent blank with a Hitachi Model 100-50 spectrophotometer.

Permeation Decomposition of Urea through Urease-Immobilized EVA Membrane

The permeation decomposition of urea was carried out at 30°C with magnetic stirring, using a diaphragm glass cell consisting of two detachable parts as shown in Figure 1. The urease-immobilized EVA membrane was set in the middle of the two parts of the cell, the left-side (L-side) chamber and the right-side (R-side) chamber of which were filled with 2.5×10^{-2} mol/L phosphate buffer (pH 8.4) of 1.3×10^{-3} mol/L urea and the buffer, respectively. Urea was decomposed into ammonia through the urease-immobilized EVA membrane, when it was diffusively transported from the L-side chamber to the R-side chamber through the membrane. The ammonia concentrations in the L-side and R-side chamber were determined by the method described above.[16] The concentrations of nondecomposed urea in the L-side and R-side were likewise obtained by the measurement of produced ammonia concentration after completely decomposing with urease.

RESULTS AND DISCUSSION

Immobilization of Urease on EVA Membrane

The methods available for the immobilization of enzyme on or within polymer supports are as follows[1–3]: (a) adsorption on the polymer support by electrostatic interaction, hydrophobic interaction, and so on; (b) entrapment by the occlusion within crosslinked gels or by encapsulation within microcapsules, liposomes, and so on; (c) covalent binding to polymer supports by the reaction of their functional groups with enzyme. In this study, we selected covalent binding so that the immobilized enzyme cannot leak out of the EVA membrane.

Urease was covalently immobilized on the EVA membrane using cyanuric chloride by the method reported by Kay and Crook[13] and Kay et al.[14] Hydroxyl groups of the EVA membrane are activated by cyanuric chloride and then the chlorine of cyanuric chloride attached to the membrane reacts with amino groups of urease, as shown in Scheme 1.

Figure 2 shows FTIR-ATR spectra of the EVA membrane at each stage of the immobilization process. In the EVA membrane activated by cyanuric chloride, the absorption peak assigned to the C=N stretching band was observed at about 1500 cm^{-1}, which cannot be found in the EVA membrane. In the EVA membrane after the reac-

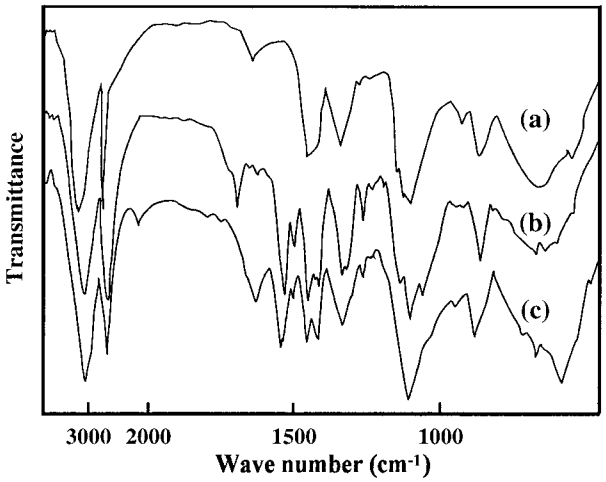


Figure 2 FTIR-ATR spectra of EVA membrane (a), cyanuric chloride-activated EVA membrane (b), and urease-immobilized EVA membrane (c).

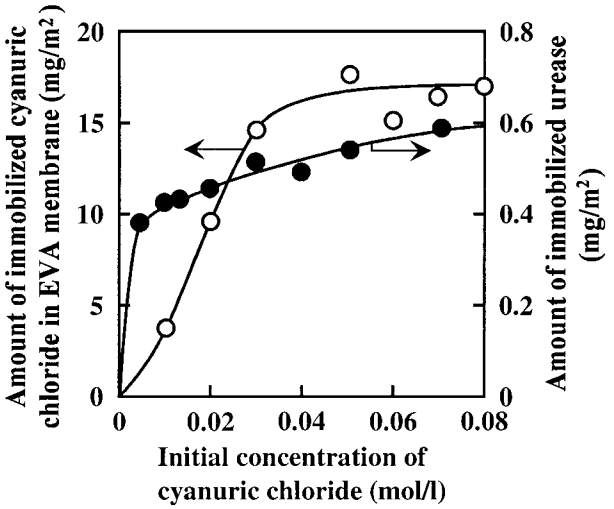


Figure 3 Effect of initial cyanuric chloride concentration on the amount of cyanuric chloride in the EVA membrane (○) and the amount of immobilized urease (●).

tion of the activated membrane with urease, the absorption peak assigned to amide group appeared at 1640 cm^{-1}. Since the urease-immobilized EVA membrane was sufficiently washed with the phosphate buffer, it is thought that urease wasn't physically adsorbed on the EVA membrane. Therefore, the absorption peak at 1640 cm^{-1} means that urease can be covalently immobilized on the EVA membrane by this method.

In an enzyme immobilization by a chemical reaction, since conformational changes in the enzyme causes inactivation due to involvement of the active site in the immobilization reaction, immobilization under the mildest conditions possible to effect immobilization is required. Next, we investigated the immobilization condition at each stage of the immobilization process to determine the optimum condition for the immobilization of urease on the EVA membrane.

Figure 3 shows the effect of initial concentration of cyanuric chloride on the amount of cyanuric chloride in the EVA membrane and the amount of immobilized urease. The amount of cyanuric chloride in the EVA membrane increased gradually with the initial concentration and became constant over 0.04 mol/L of the initial concentration. Accordingly, the amount of immobilized urease increased drastically with a small amount of initial cyanuric chloride, but showed a slow increase with more than 0.01 mol/L.

The relationship between the specific activity of the resulting urease-immobilized EVA membrane and the initial concentration of cyanuric chloride is shown in Figure 4. The specific activity exhibited a maximum at the initial concentration of about 0.015 mol/L and became approximately constant over 0.02 mol/L. Kay and Crook[13] and Kay et al.[14] reported that cyanuric chloride in the vicinity of immobilized urease inhibits the enzyme activity. As can be seen from Figure 3, at the initial concentration of cyanuric chloride more than 0.02 mol/L, a sharp increase in the amount of cyanuric chloride in the EVA membrane and a slow increase in the amount of immobilized enzyme lead us to the conclusion that there is a large amount of cyanuric chloride which does not react with urease. Therefore, the decrease of the specific activity over the initial concentration of 0.02 mol/L is attributed to the inhibition of excess cyanuric chloride in the vicinity of immobilized urease. Consequently, the optimum proportion of cyanuric chloride in the membrane and immobilized urease results in maximum specific activity at the initial cyanuric chloride concentration of 0.015 mol/L.

Figure 5 shows the effect of initial urease concentration on the amount of urease immobilized on the EVA membrane and its specific activity. Since cyanuric chloride in the EVA membrane becomes constant over the initial concentration of 0.04 mol/L (Fig. 3), the initial cyanuric chloride concentration was fixed at 0.04 mol/L. An increase in the initial urease concentration gave rise

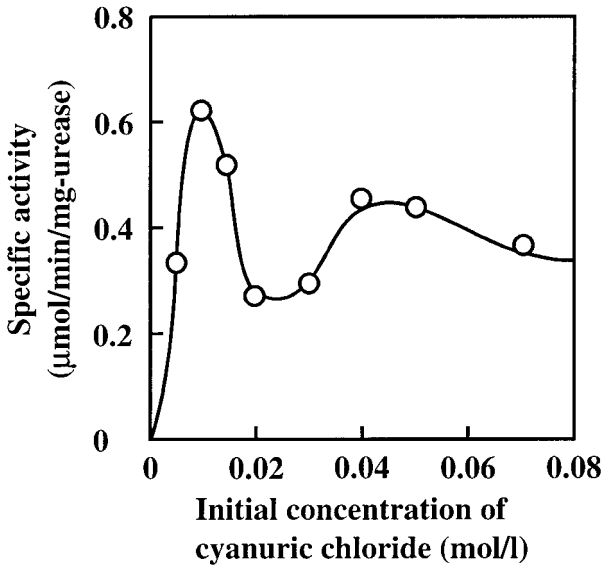


Figure 4 Effect of initial cyanuric chloride concentration on the specific activity of urease-immobilized EVA membrane at pH 7.0, 30°C. Initial urease concentration; 0.4 g/L.

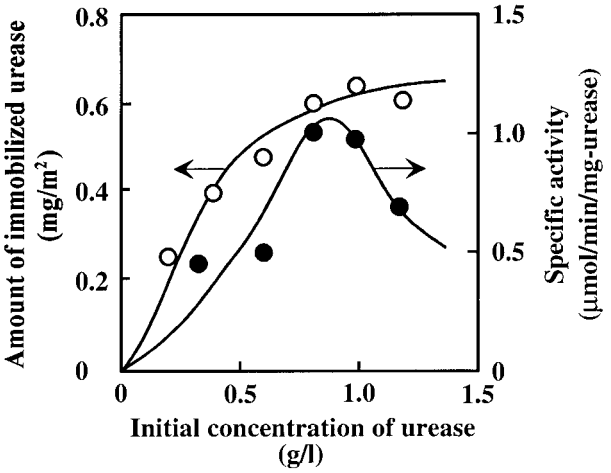


Figure 5 Effect of initial urease concentration on the amount of immobilized urease and its specific activity for decomposition of urea at pH 7.0, 30°C. Initial cyanuric chloride: 0.04 mol/L.

to a gradual increase in the amount of immobilized urease. The specific activity of urease-immobilized EVA membrane exhibited a maximum at the initial urease concentration of 0.8 g/L. The maximum specific activity is only 10% of that of the native urease. When immobilization of enzyme is accompanied by chemical reactions such as crosslinking and covalent binding methods, conformational change in the enzyme causes inactivation due to involvement of the active site in the immobilization reaction. Therefore, the low specific activity of the urease-immobilized EVA membrane is due to a conformational change of urease during the immobilization. Furthermore, although native urease catalyzes the decomposition of urea in homogeneous solution, the diffusion of the substrate into the membrane may be a rate-determining step in the decomposition by the urease-immobilized EVA membrane. The slow diffusion of the substrate may cause the apparently low specific activity of the urease-immobilized EVA membrane. On the other hand, total activity exhibits a maximum at the initial urease concentration of about 1.0 g/L as a result of both maximum specific activity at 0.08 g/L and gradual increase in the amount of immobilized urease on the EVA membrane. Therefore, we hereinafter selected the initial urease concentration of 1.0 g/L as an immobilization condition.

Enzyme Activity of Urease-Immobilized EVA Membrane

Figure 6 shows the influence of pH on the relative activity of the urease-immobilized EVA membrane and native urease. The optimum pH of the urease-immobilized EVA membrane is about 7.0, similar to that of native urease. Their activity is low except at pH 7.0. Enzymes are well known to be active in only a limited pH range. An optimum pH for enzyme activity usually appears, because enzymes have many ionizable groups and pH changes may give rise to changes in the conformation of the enzyme, binding of the substrate, and the catalytic activity of groups in the active site of the enzyme.[1–3] As can be seen from this figure, furthermore, the width of the pH dependence of relative activity is broader for the immobilized urease than for native urease. In many cases, the immobilization of an enzyme improves the stability of the enzyme structure.[17] Therefore, the immobilization of urease on the EVA membrane may inhibit the conformational change of urease by pH changes.

An advantage of immobilized enzyme is the repeated use without troublesome separation of enzyme from the product. Activity decay in repeated use is often observed in some immobilized enzymes. The results for up to 5× repeated use of the urease-immobilized EVA membrane in a batch reaction are shown in Figure 7. Each enzyme reaction was carried out in 2.5×10^{-2} mol/L phosphate buffer containing 1.3×10^{-2} mol/L urea at 30°C for 10 min. The urease-immobilized EVA membrane exhibited no activity decay dur-

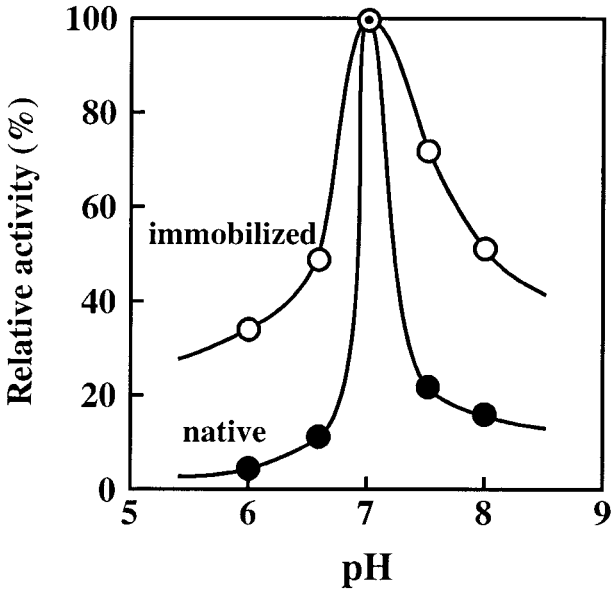


Figure 6 pH dependence of relative activity of the urease-immobilized EVA membrane (○) and native urease (●).

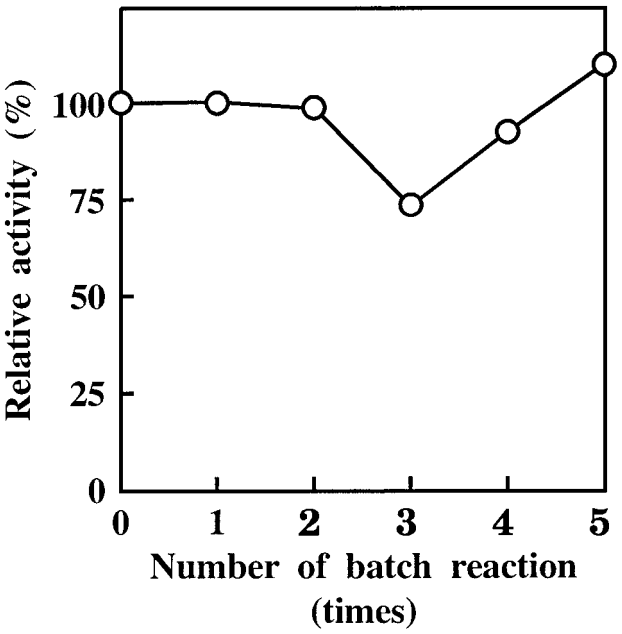


Figure 7 Stability for repeated use of the urease-immobilized EVA membrane. Each enzymatic reaction was carried out at 30°C for 10 min.

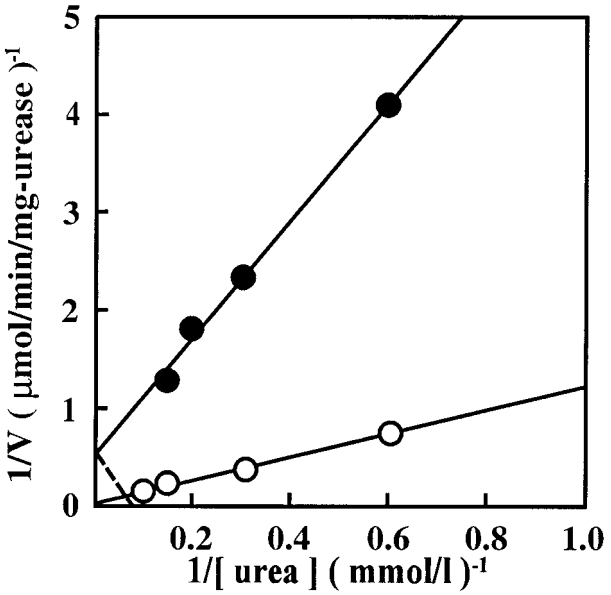


Figure 8 Lineweaver-Burk plots of native urease (○) and urease-immobilized EVA membrane (●). The enzymatic reaction (decomposition of urea) was carried out in 0.025 mol/L phosphate buffer (pH 7.0) at 30°C for 10 min.

ing the repeated use of 5×. This indicates that the immobilized urease isn't inactivated and does not leak out of the EVA membrane during the repeated use. Thus, the activity measurements lead us to the conclusion that the urease-immobilized EVA membrane is effective for the decomposition of urea due to stability of the activity, easy separation from the products, and the stable repeated use.

Enzyme kinetics are best described by the well-known Michaelis–Menten equation on the basis of the complex formation between an enzyme and a substrate as follows:[1–3,18]

$$v = \frac{V_m[S]}{K_m + [S]} \quad (1)$$

The values of K_m and V_m can be derived graphically following a transposition of the Michaelis–Menten equation to give:

$$\frac{1}{v} = \frac{K_m}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m} \quad (2)$$

The reciprocal of the velocity ($1/v$) is plotted as a function of the reciprocal of the initial substrate concentration ($1/[S]$) in Figure 8 (Lineweaver-Burk plot). The linear plots for the urease-immobilized EVA membrane demonstrate that the decomposition of urea by the membrane is apparently of the Michaelis-Menten type. The intercepts with the $1/[S]$ and $1/v$ axes give the values of $1/K_m$ and $1/V_m$, respectively, and the slope of the line is K_m/V_m. The Michaelis constant (K_m) and maximum velocity (V_m) of native urease and the urease-immobilized EVA membrane are shown in Table I. The K_m of the urease-immobilized EVA membrane was smaller than that of native urease. In enzymatic processes, K_m indicates the reciprocal affinity of an enzyme and a substrate.[19] The smaller K_m of the urease-immobilized EVA membrane may mean that the complex between immobilized urease and a substrate is formed easily. On the other hand, V_m of the ure-

Table I Kinetic Data for Decomposition of Urea by Native Urease and Immobilized Urease on EVA Membrane

	K_m (mmol/L)	V_m (μmol/min/mg)
Native urease	29.95	36.13
Immobilized urease	11.96	1.98

ase-immobilized EVA membrane was much smaller than that of native urease. Since the decomposition of urea by the urease-immobilized EVA membrane is heterogeneous, the apparent velocity of the decomposition is influenced by the diffusion of urea into the membrane. The smaller V_m of the urease-immobilized EVA membrane compared with native urease is thought to be attributable to the heterogeneous catalysis and inactivation of urease during the immobilization.

Permeation Decomposition of Urea through Asymmetrically Urease-Immobilized EVA Membrane

Previous papers made it clear that there is a significant difference in the amount of hydroxyl groups between the water side surface and polyethylene (PE) side surface in EVA membranes prepared on the PE plate by the phase inversion method.[9,10] We also clarified that the catalyst immobilized in the EVA membrane is asymmetrically distributed in the direction of the membrane cross section.[11,12] The asymmetric distribution of catalyst is effective for the improvement of the membrane performance such as the separation of products. Furthermore, Ciftci and Vieth[20] theoretically predicted that the reaction efficiency and the separation of products depends upon asymmetric distribution of enzyme in a reactor.

In this study, we tried to prepare a urease-immobilized EVA membrane in which urease is asymmetrically distributed, as follows: After the EVA membrane was prepared by casting the polymer solution on the PE plate and dipping it into water, it was activated by cyanuric chloride without the membrane being taken off the PE plate. This procedure can lead to the activation of the EVA membrane from only the water side surface by cyanuric chloride. The asymmetrically urease-immobilized EVA membrane may be prepared by the immobilization of urease on the resulting cyanuric chloride-activated EVA membrane.

Figure 9 shows FTIR-ATR spectra of the urease-immobilized EVA membrane prepared by the method described above. A significant difference in the spectra is found between the PE side surface and the water side surface of the membrane. Although the absorption peak related to urease was observed at about 1700 cm^{-1} on the water side surface of the urease-immobilized EVA membrane, the peak didn't appear on the surface of the PE side. This indicates that urease is immobilized on only the water side surface of the EVA membrane, because the EVA membrane is asymmetrically activated by cyanuric chloride without being taken off the PE plate. Consequently, an asymmetrically urease-immobilized EVA membrane can be prepared by this method.

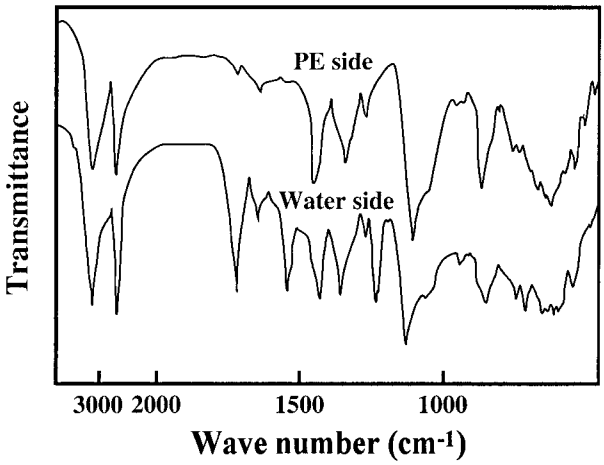


Figure 9 FTIR-ATR spectra of the PE side and water side surface of urease-immobilized EVA membrane cast on a PE plate.

In order to reveal the relationship between the asymmetric distribution of urease and the permeation decomposition of urea through the urease-immobilized EVA membrane, urea was decomposed through the membrane by two methods as follows: The membrane was set with the water side surface of the membrane, at which more urease is immobilized than at the PE side surface, facing the left-side (L-side) or the right-side (R-side) chamber of the diaphragm glass cell. We call the former and the latter Method I and Method II, respectively.

Figures 10 and 11 show time dependence of the nondecomposed urea and decomposed urea concentrations in both side chambers of the diaphragm cell during the permeation decomposition by Method I and II, respectively. Urea was decomposed into ammonia through the urease-immobilized EVA membrane by these methods. The amount of decomposed urea in the R-side was smaller than that in the L-side. This is attributed to the higher concentration of the substrate in the L-side than in the R-side, because the reaction velocity of the Michaelis-Menten type is dependent upon the substrate concentration. It is obvious that the nondecomposed urea and decomposed urea concentration in permeation by

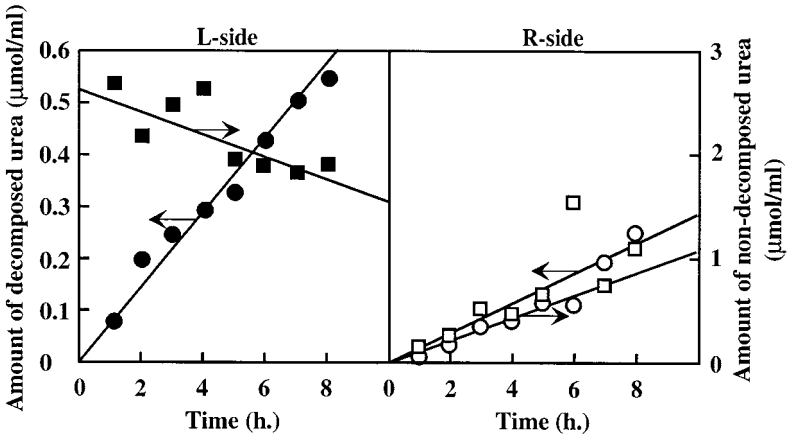


Figure 10 Time dependencies of the decomposed urea concentration (○, ●) and non-decomposed urea concentration (□, ■) in the L-side and R-side chamber of diaphram cell, when urea was decomposed through the urease-immobilized EVA membrane in 0.025 mol/L phosphate buffer (pH 7.0) at 30°C. The water side surface of the urease-immobilized EVA membrane was faced to the L-side chamber (Method I).

Method I differed from those by Method II. In the R-side, by Method I, the concentration of decomposed urea through the urease-immobilized EVA membrane was approximately the same as that of the nondecomposed urea. In the R-side, by Method II, however, the concentration of decomposed urea through the membrane was higher than that of nondecomposed urea. The asymmetrically urease-immobilized EVA membrane suppressed the permeation of the nondecomposed urea, and more efficient separation of a product (ammonia) from a reactant (urea) was achieved by Method II than by Method I. The tendency of the difference between Method I and Method II is similar to that in the permeation oxidation of hydroquinone through an EVA–Cu complex membrane[12] and in the permeation hydrolysis of *p*-nitrophenyl acetate through a CD-immobilized EVA membrane reported in our previous paper.[11] This tendency is supported by theoretical prediction by Ciftci and Vieth.[20] The asymmetric distribution of immobilized urease in the EVA mem-

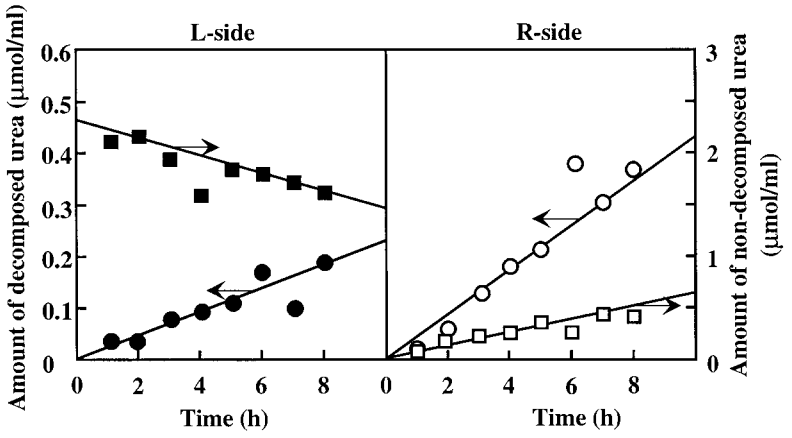


Figure 11 Time dependencies of the decomposed urea concentration (○, ●) and non-decomposed urea concentration (□, ■) in the L-side and R-side chamber of diaphram cell, when urea was decomposed through the urease-immobilized EVA membrane in 0.025 mol/L phosphate buffer (pH 7.0) at 30°C. The water side surface of the urease-immobilized EVA membrane was faced to the R-side chamber (Method II).

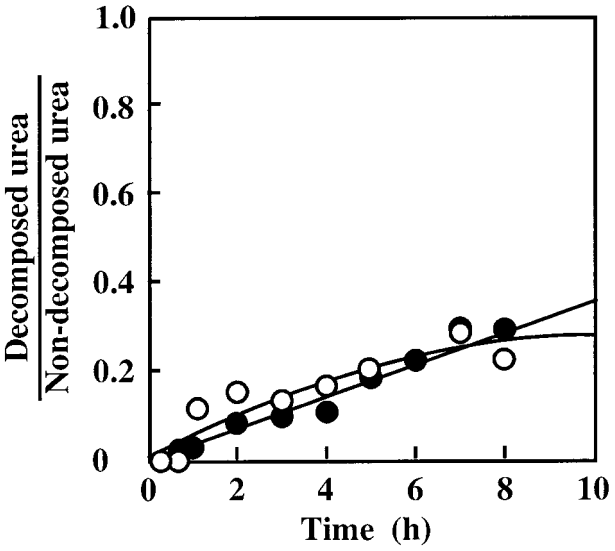


Figure 12 Time dependencies of the ratio of decomposed urea and nondecomposed urea in the L-side chamber (●) and the R-side chamber (○) of diaphram cell, when urea was decomposed through the urease-immobilized EVA membrane in 0.025 mol/L phosphate buffer (pH 7.0) at 30°C. The water side surface of the urease-immobilized EVA membrane was faced to the L-side chamber (Method I).

brane causes the difference in the permeation reaction between Method I and Method II. Generally, a high-performance membrane reactor should have both a high catalytic action and an excellent separation of products. In order to evaluate the membrane performance as a reactor, we calculated the ratio of decomposed urea to nondecomposed urea on both sides. The results, as a function of time by Method I and II, are shown in Figures 12 and 13, respectively. The ratio of decomposed urea to nondecomposed urea by Method II was much larger in the R-side than the L-side, in contrast to Method I. Namely, Method II led to the suppression of the permeation of nondecomposed urea and the separation of decomposed urea from nondecomposed urea. Consequently, Method II is more effective for the permeation decomposition of urea through the asymmetrically urease-immobilized EVA membrane than Method I.

CONCLUSIONS

Urease was covalently immobilized on an ethylene–vinyl alcohol copolymer (EVA) membrane activated with cyanuric chloride. The optimum condition for immobilization was determined by investigation of the effects of the initial cyanuric chloride and the initial urease concentration on the amount of immobilized urease and the specific activity. The urease-immobilized EVA membrane showed the same optimum pH for activity as native urease and held a high relative activity over a wider range of pH. When the urease-immobilized EVA membrane was repeatedly used in the decomposition of urea, the relative activity of the membrane didn't decay. The Michaelis constant and the maximum velocity of the urease-immobilized EVA membrane were determined on the basis of Michaelis-Menten kinetics. FTIR-ATR spectra demonstrated that the immobilization of urease on the EVA membrane prepared on a polyethylene plate led to membrane in which urease was asymmetrically distributed in the direction of membrane cross section. The permeation decomposition of urea through the asymmetrically urease-immobilized EVA membrane was examined by two methods for setting the membrane in a diaphragm glass cell. Consequently, the asymmetric distribution of urease in the EVA

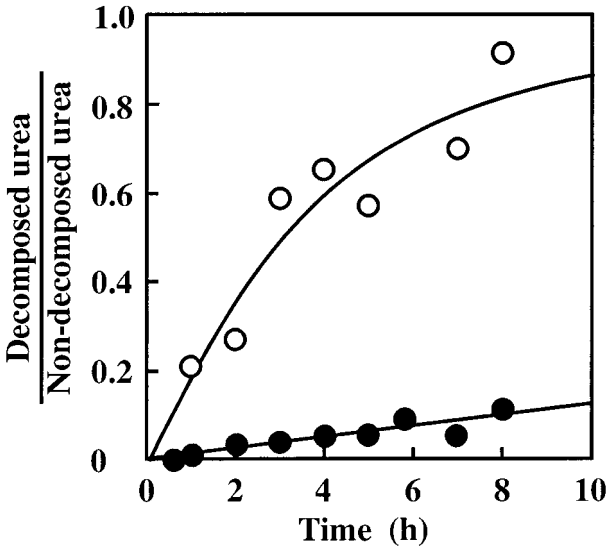


Figure 13 Time dependencies of the ratio of decomposed urea and nondecomposed urea in the L-side chamber (●) and the R-side chamber (○) of diaphram cell, when urea was decomposed through the urease-immobilized EVA membrane in 0.025 mol/L phosphate buffer (pH 7.0) at 30°C. The water side surface of the urease-immobilized EVA membrane was faced to the R-side chamber (Method II).

membrane influenced the membrane performance (the separation of products) as a reactor.

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