Epoxy-Derived pHEMA Membrane for Use Bioactive Macromolecules Immobilization: Covalently Bound Urease in a Continuous Model System

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ABSTRACT: Poly(2-hydroxyethylmethacrylate) (pHEMA) membranes were prepared by UV-initiated photopolymerization of HEMA in the presence of an initiator (α-α'-azobis-isobutyronitrile, AIBN). The epoxy group, i.e., epichlorohydrin, was incorporated covalently, and the urease was immobilized onto pHEMA membranes by covalent bonding through the epoxy group. The retained activity of the immobilized enzyme was found to be 27%. The K_m values were 18 and 34 mM for the free and the immobilized enzymes, respectively, and the V_{max} values were found to be 59.7 and 16.2 U mg^{-1} for the free and the immobilized enzyme. The optimum pHs was 7.2 for both forms, and the optimum temperature for the free and the immobilized enzymes were determined to be 45 and 50°C, respectively. The immobilized urease was characterized in a continuous system and during urea degradation the operational stability rate constant for the immobilized enzyme was found to be 5.83×10^{-5} min^{-1}. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 77: 2000–2008, 2000

Key words: covalent bonding; enzyme immobilization; urease; enzyme reactor; pHEMA membrane

INTRODUCTION

Immobilization technology is becoming an important field in the biomedical science and biotechnology. A large number of bioactive materials such as drugs, proteins, plant, and animal cells, and micro-organisms of various classes were successfully immobilized with very high yields on appropriate supports.[1–3] These immobilized products were intended for use in the construction of artificial organ systems, biosensors, or bioreactors.[1,4] Immobilization is advantageous because (1) it extends the stability of the bioactive species by protecting the active material from deactivation, (2) it enables repeated use, (3) it provides significant reduction in the operation costs, (4) it facilitates easy separation and speeds up recovery of the bioactive agent.[5] The availability of a large number of support materials and methods of immobilization leave virtually no bioactive species without a feasible route of immobilization. It is, thus, important that the choice of support material and immobilization method over the free bioactive agent should be well justified.[6–9]

Suitable matrices include hydrogels that are highly compatible for immobilization of enzymes due to their hydrophilic nature. Poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels are among the major synthetic polymers approved by federal agencies (like the Food and Drug Administration in the USA) for biomedical and pharmaceutical applications.[10] pHEMA is a nontoxic and biocompatible synthetic polymer with adequate

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mechanical strength for most biomedical applications. In addition, it contains hydroxyl groups that act as attachment sites for bioactive species after activation.[8–11] pHEMA can easily be prepared in different forms such as microspheres and membranes. A membrane form has a high possibility as an excellent support for enzyme immobilization, because high reaction efficiency and simple separation of products can be achieved using enzyme membranes.

Urease converts urea to ammonium ion and carbon dioxide. The ammonium ions produced by enzyme-catalyzed urea hydrolysis are toxic in humans at blood levels above 10^{-4} mol L^{-1}.[12–14] Urease immobilized membrane can be used in biosensors or especially in artificial kidney devices for the removal of urea from blood for extracorporeal detoxification.[4,15,16] An artificial kidney is mainly composed of a membrane (hemodialyzer) that separates blood from dialysate solution through the membrane. Various types polymeric materials such as cellulose, polyacrylonitrile, or an ethylene–vinylalcohol copolymer are used in the membrane form for the hemodialyzer.[14] The pHEMA membrane could be also expected to be useful for the hemodialyzer.

Several methods have been developed for the preparation of immobilized urease; each having its own advantages and disadvantages.[4,14,16,17] After immobilization, changes were observed in enzymatic activity, optimum pH and temperature, affinity to substrate, and stability. Generally, the changes in the enzymatic activity and in the affinity to substrate were unfavorable, whereas the optimum temperature and the stability were improved. The extent of these changes depended on the nature of enzyme, type of support, and on the immobilization conditions.[18–22]

In this study the aim was to immobilize urease on the biocompatible hydrogel support with a significantly higher stability than those obtained in earlier studies. Urease was, therefore, covalently immobilized onto the pHEMA membrane, and the effect of various parameters on enzyme activity was studied. Finally, immobilized urease was applied to an enzyme reactor to study the behavior of the enzyme in a continuous system.

EXPERIMENTAL

Materials

Urease (EC 3.5.1.5 from Jack Beans, Type III), bovine serum albumin (BSA), and urea kit (Bun color reagent; trichloroacetic acid solution (3.0% w/v), diacetyl monoxime solution (0.18% w/v), Bun acid reagent and urea nitrogen standard solution) were all obtained from the Sigma Chemical Company (St. Louis, MO). $\alpha\alpha'$-Azoisobutyronitrile (AIBN) and 2-hydroxyethyl methacrylate (HEMA) were obtained from Fluka AG (Switzerland), the later was vacuum distilled in the presence of hydroquinone inhibitor and stored in the refrigerator until use. All the other analytical grade chemicals were purchased from Merck AG. (Darmstadt, Germany).

Membrane Preparation and Immobilization

The membrane preparation mixture (5 mL) contained 2 mL (HEMA), 5 mg AIBN as polymerization initiator, and 3 mL phosphate buffer (0.1 *M*, pH 7.0). The mixture was then poured into a round glass mold (diameter: 9.0 cm) and exposed to ultraviolet radiation for 10 min, while a nitrogen atmosphere was maintained in the mold. The membrane was washed several times with distilled water and cut into circular pieces (diameter: 1.0 cm) with a perforator.

The activation of alcoholic groups of pHEMA membrane for covalent immobilization of urease

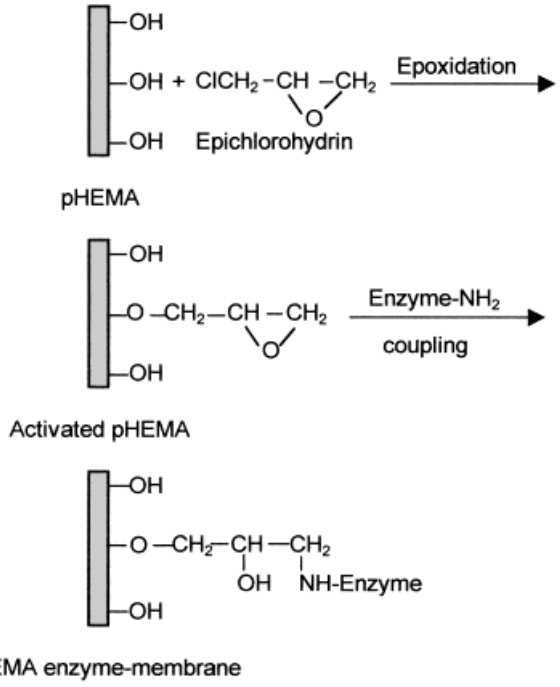

Figure 1 Activation of the pHEMA with epichlorohydrin and covalent immobilization of the enzyme.

was achieved by reaction with epichlorohydrin (Fig. 1). Epoxy groups were covalently incorporated to the pHEMA membrane via the nucleophilic reaction between the chloride group of epichlorohydrin and hydroxyl group of the HEMA molecule under alkaline conditions. The pHEMA membrane disks (diameter 1.0 cm, thickness about 0.06 cm, 15 g, total surface area about 200 cm^2) were immersed in a reactor containing NaOH solution (4.0% (w/v), 25 mL) for 1 h. The epicholorohydrin (50 mL) was then added and was stirred magnetically at 25°C for 6 h. At the end of this period, pHEMA membrane disks were then washed with acetone and dried at 4°C. The membrane disks were kept in dry form at 4°C until use.

Activated pHEMA membrane disks were swollen in phosphate buffer (0.1 *M*, pH 7.5) for 18 h, was then transferred to the enzyme solution (30 mg urease in 30 mL phosphate buffer) in a batch system. Immobilization reaction was carried out at 22°C in a shaking water bath. To optimize the extent of enzyme immobilization, the coupling duration time was varied between 6 and 24 h. Ionically bound enzyme was removed first by washing the supports with saline solution (10 mL, 0.5 *M*) and then with phosphate buffer (20 m*M*, pH 7.0), and was stored at 4°C in fresh buffer until use.

Determination of Immobilization Efficiency

The amount of protein in the enzyme solutions was determined using Coomassie Brilliant Blue as described by Bradford[23] with bovine serum albumin (BSA) as a standard.

Urease Activity Measurements

The determination of the activities of the free and immobilized urease was carried out according to the procedure given in Sigma Blood Urea Nitrogen Kit (Sigma Catalog No: 535). For the construction of a calibration curve Bun acid reagent (3.0 mL), Bun color reagent (2.0 mL) and urea nitrogen solutions (20 μL, 2–20 m*M* urea) were transferred to a series of test tubes and were maintained for exactly 10 min in boiling water. After cooling, the absorbances at 525 nm were determined with a spectrophotometer (Shimadzu Model 1601, Japan).

Free Enzyme

Urea solution (4.9 mL) (3–25 m*M* urea in phosphate buffer pH 7.2) were preincubated at 35°C for 10 min, and the reaction was started by adding 0.1 mL of enzyme solution (1 mg urease per mL). After the enzymatic reaction a 20 μL aliquot was transferred to a solution consisting of Bun color (2.0 mL) and Bun acid reagent (3.0 mL) at specific time intervals. The decrease of urea concentration in solutions were measured as described above.

Immobilized Enzyme

For the determination of immobilized urease activity, five hydrogel disks (surface area about 8.0 cm^2) were introduced to the urea nitrogen solution (10 m*M*, 5 mL), and the decay of urea was followed as above. The activity of the immobilized urease was presented as a percentage of the activity of free enzyme of same quantity. One unit of urease activity is defined as the amount of enzyme, which liberate 1.0 μmol NH_3, from urea per min at 35°C at pH 7.2.

Dependence of Enzyme Activity on pH and Temperature

The effect of pH on the activity of free and immobilized enzyme was investigated in a batch system at 35°C. A urea concentration (10 m*M*) was prepared in acetate buffer (0.1 *M*) in the pH range of 4.0–5.5 and in phosphate buffer (0.1 *M*) in the range of pH 6.0–8.0. The effect of temperature on enzymes activity was also studied in the range of 20–60°C with a urea concentration was 10 m*M* in phosphate buffer (0.1 *M*, pH 7.2).

Packed Bed Reactor and Operation

The reactor (length 9 cm, diameter 2 cm, total volume 28 mL), was made from Pyrex® glass. The enzyme membrane disks were equilibrated in phosphate buffer (0.1 m*M*, pH 7.2) at 4°C for 1 h. Enzyme membrane disks (15 g) (total area ca. 200 cm^2 and 110 U) were loaded into the reactor yielding a void volume of about 13 mL.

To determine the effect of substrate concentration on reactor productivity, urea solution (5–45 m*M*) in the phosphate buffer was introduced to the reactor at a rate of 60 mL h^{-1} with a peristaltic pump (Cole Parmer, Model 7521-00, Miles, IL) through the lower inlet part. The solution leaving the reactor was collected in a fraction collector and was assayed for urease activity at the end of each hour.

The performance of immobilized urease in the bed reactor can be described under steady-state conditions by means of the integrated form of the Michaelis-Menten equation.[24]

$$-d[R]/dt = V_{\max}[R]/(K_m + [R]) = V_{\max}/((K_m/[R]) + 1) \quad (1)$$

where $[R]$ is the reactant concentration (M) in the reactor; $V_{\max}$ is the maximum rate of reaction (M s^{-1}); K_m is the Michaelis constant (M); and t is time (seconds).

For a bed reactor an integrated equation can be written by replacing the reaction time with residence time, V_{tot}/Q (where V_{tot} is the reactor volume (dm^3) and Q is the volumetric flow rate (dm^3s^{-1}), the time that each fluid element spends in the reactor. The voidage of the reactor can be expressed as $\epsilon = V_1/V_{tot}$, V_1 is the volume of the enzyme membrane in the system (dm^3), and eq. (1) can be rearranged as:

$$V_{\max} \cdot V_{tot} \cdot \varepsilon/Q = K_m \cdot \ln([R_0]/[R]) + ([R_0] - [R]) \quad (2)$$

where $[R_o]$ is the reactant concentration in the feed (M). The performance equation for the enzyme reactor can be rearranged for the calculation of the kinetic constants from experimental data.

$$[R_0] \cdot X = K_m \cdot \ln(1 - X) + V_{\max} \cdot V_{tot} \cdot \varepsilon/Q \quad (3)$$

where X is defined as $([R_o] - (R])/[R_o]$.

Thus, plotting experimentally obtaining values of $[R_o]$ X vs. ln $(1-X)$ will give a graph having a slope of K_m and an intercept of $V_{\max} \cdot V_{tot} \cdot \epsilon/Q$.

The effect of flow rate on reactor performance was studied by varying the flow rate in the range of 30–100 mL h^{-1} at 35°C for 2 h, while keeping the concentration of urea at 30 mM in phosphate buffer (0.1 M, pH 7.2).

To determine operational stability of immobilized urease, the reactor was loaded with immobilized urease and operated at 35°C for 40 h. The feed solution was contained urea (10 mM) in phosphate buffer (0.1 M, pH 7.2) with a flow rate of 60 mL h^{-1}. Enzyme activity in the solution leaving the reactor was measured as described above.

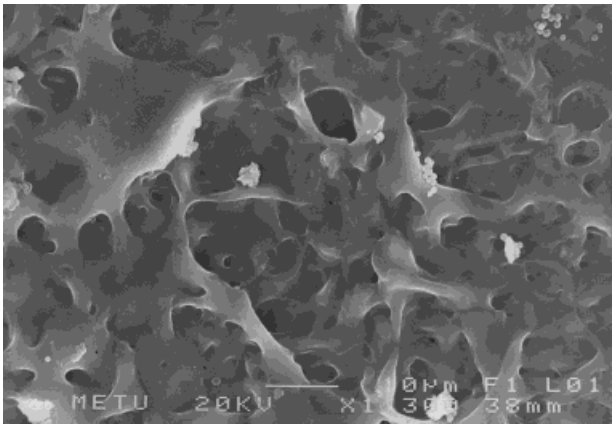

Figure 2 SEM of the pHEMA membrane surface.

Thermal Stability Measurements of Free and Immobilized Enzymes

Thermal stability of the free and immobilized urease were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (45–65°C) in phosphate buffer (0.1 M, pH 7.2) for 2 h. A sample was removed at a 15-min time interval and assayed for enzymatic activity. The first-order inactivation rate constant, k_i, was calculated from the equation:

$$\ln A = \ln A_0 - k_i t \quad (4)$$

where A_0 and A are the initial activity and the activity after time t (min).

Scanning Electron Microscopy

Scanning electron micrographs of the freeze-dried pHEMA membrane were obtained using a Leittz AMR-1000 microscope (Germany) after coating with gold under vacuum.

RESULTS AND DISCUSSION

Immobilization of Urease and Kinetic Constants

The activation of alcoholic groups of the pHEMA membrane was achieved by reaction with epichlorohydrin under alkaline conditions (Fig. 1). Urease was then covalently immobilized via the amino group to the epoxy groups of the activated pHEMA membrane. The SEM pictures of the pHEMA membrane (Fig. 2) show that the mem-

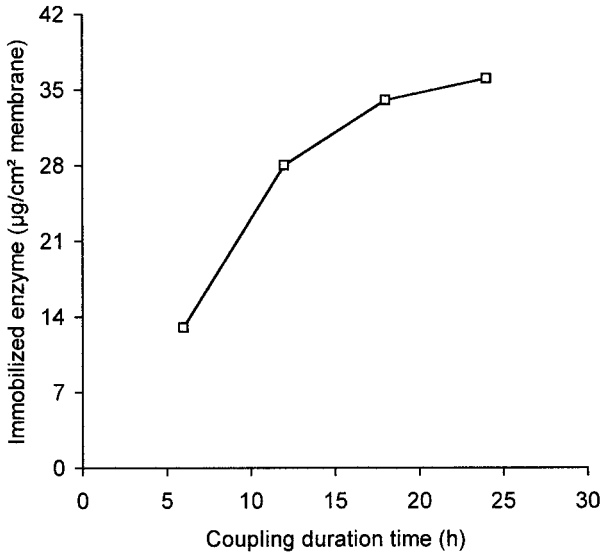

Figure 3 Coupling time vs. amount of the immobilized enzyme.

brane has a highly open pore structure, which may lead to high internal surface area (implying high immobilization capacity). The enzyme should be immobilized both the external surface of the membrane and within the pore space near the surface, and thus provided a large surface area for the reaction of substrate with the immobilized enzyme. Analysis of wash solutions showed that the immobilization process was irreversible. As observed in Figure 3, an increase in coupling time led to an increase in extent of immobilization, but this relation leveled off after 18 h. Thus, a maximum enzyme loading of 34 μg cm^{-2} was obtained, and the covalently bound enzyme retained about 27% of its initial activity.

Kinetic parameters, the Michaelis constant K_m and the $V_{\max}$ for the free and the immobilized urease were determined using urea as a substrate (Table I). K_m value for the free enzyme was found to be 18 mM and the value of apparent K_m for covalently immobilized urease was (34 mM), approximately twofold higher than that of the free enzyme. In a similar study, the K_m value of the immobilized urease was fourfold higher than that of the free urease due to the influence of the support, which had a highly ionic character.[4] The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme. The $V_{\max}$ value for the free enzyme was 59.7 U mg^{-1} protein. The $V_{\max}$ value was also significantly decreased (16.2 U mg^{-1}) upon covalent immobilization of urease on the membrane. Several researchers reported that $V_{\max}$ values of enzymes showed a decrease upon immobilization.[4,12–15,25,26]

Effect of pH and Temperature on Activity

The effect of pH on the activity of free and immobilized urease in urea degradation was carried out in the pH range of 4.0–9.0, and the results are presented in Figure 4. From this figure, in very acidic or alkaline regions, the activity of both free and immobilized preparations were decreased. Optimal conversion was obtained at pH 7.2 for both the free and immobilized form. The immobilized urease approximately has almost the same pH activity profile with that of the free enzyme, except that the pH profile of the immobilized enzyme was broadened in the alkaline region. This observed displacement toward to the alkaline region for the immobilized enzyme is because pH conditions in the pore space of polymeric matrix are different from those in the rest of the solution. The enzymatic product (amonium ions) is a charged molecule, which will effect the local pH. Also, the polar hydroxyl groups of the pHEMA

Table I Properties of the Free and the Immobilized Urease on the pHEMA Membrane

Enzymes	K_m (mM)	$V_{\max}$ (U mg enzyme^{-1})	Loading (μg cm^{-2})	Recovered Activity (%)
Free enzyme	18	59.7	—	100
Immobilized enzyme	34	16.2	34	27

A standard curve was prepared with urea solutions of different concentration and the slope of the curve was used in the quantification of urea in the sample.

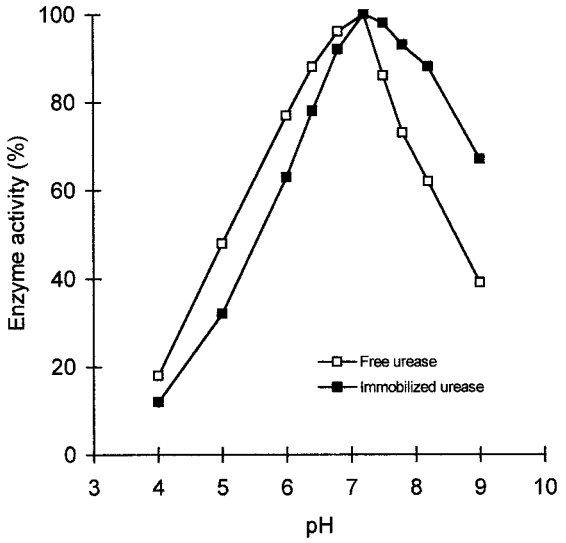

Figure 4 pH profiles of the free and the immobilized urease.

membrane may have interact with functional groups of the urease (e.g., polar interaction and hydrogen bonding) changing the pH characteristic of the enzyme. Other researchers have reported similar observations upon immobilization of urease and other enzymes.[12–15,27–30]

The temperature dependence of the activities of the free and immobilized urease was studied in the temperature range of 20–60°C (Fig. 5). The data revealed bell-shaped curves with optimum activity at 45°C for the free and at 50°C for the immobilized enzymes. A plateau region is seen for immobilized enzyme at temperature between 50 and 55°C that was not apparent for the free enzyme. Arrhenius plots in the temperature range from 20°C to optimum appear linear, and activation energies were found to be 1.47 kcal mol^{-1} and 1.83 kcal mol^{-1} for the free and the immobilized urease, respectively. The increase in optimum temperature and activation energy was caused by the changing physical and chemical properties of the enzyme. The covalent bond formation via amino groups of the immobilized enzyme might also reduce the conformational flexibility, and may result in higher activation energy for the molecule to reorganize to the proper conformation for the binding to substrate.[25]

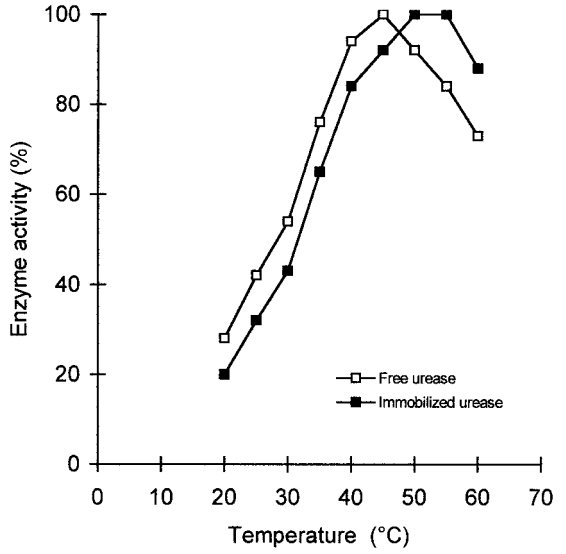

Figure 5 Temperature profiles of the free and the immobilized urease.

Reactor Productivity

The effect of substrate concentration on reactor productivity was determined by using various urea concentrations. A linear increase in degradation rate was observed up to urea concentration of 30 m*M* (Fig. 6). Beyond this urea concentration a steady level in the degradation rate was observed. This decrease in degradation rate could be either due to insufficient contact duration or toxic product (ammonia) formation does not produce

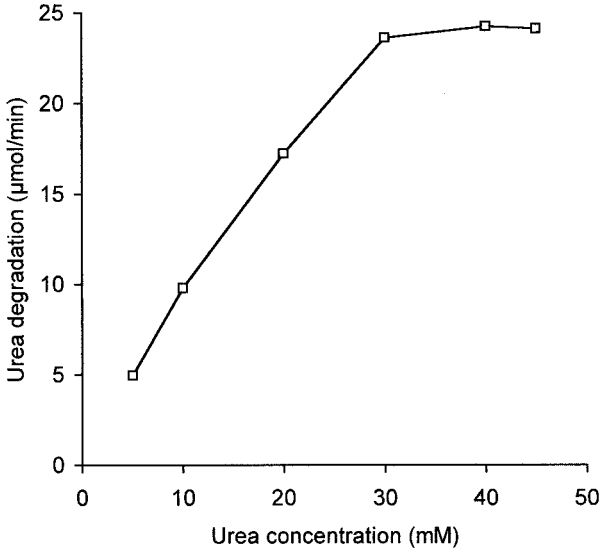

Figure 6 Effect of substrate concentration on the reactor performance. Operation condition: loading 110 U/200 cm^2 enzyme membrane; urea concentration: 5–45 m*M*; flow rate: 60 mL/h; pH: 7.2; temperature: 35°C.

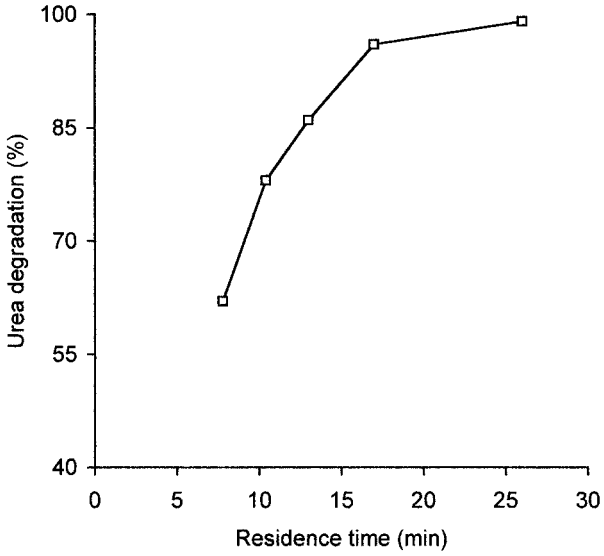

Figure 7 Influence of residence time on degradation rate for the immobilized urease in the packed bed reactor. Operation condition: loading 110 U/200 cm^2 enzyme membrane; urea concentration: 30 m*M*; flow rate: 30–100 mL/h; pH: 7.2; temperature: 35°C.

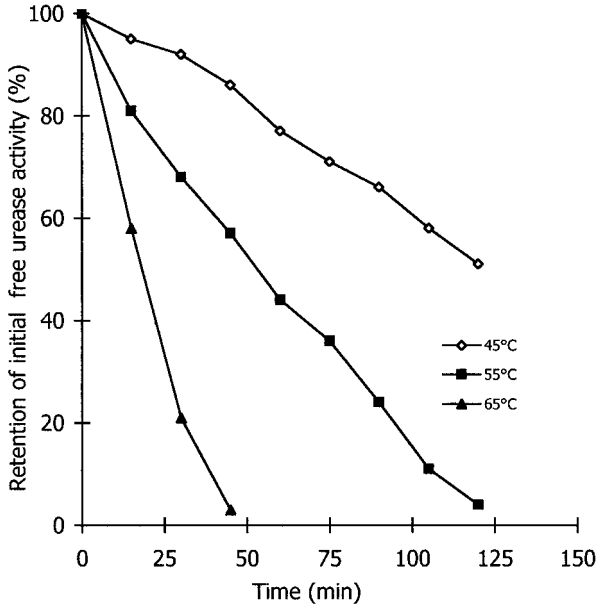

Figure 8 Influence of temperature on the stability of free urease.

higher degrees of urea degradation. Complete degradation was expected even with 45 m*M* urea and at 60 mL h^{-1} flow rate because the reactor was sufficiently loaded with about 110 U/200 cm^2 of the enzyme membrane.

Figure 7 shows the effect of residence time on degradation of urea by urease. The results were converted to relative activities (percentage of the maximum degradation obtained in this series). As the residence time is increased, the extent of degradation of urea is also increased (to almost complete degradation). Eventually, at a contact duration of about 17 min a plateau is reached (obtained with a flow rate 45 mL h^{-1} and 30 m*M* substrate).

The operational stability of covalently immobilized urease was studied in the packed bed reactor for 40 h. It was observed that immobilized urease lost only about 5% of its activity after 20 h of the continuous operation. At the end of the 40-h operation only about 13% activity was lost. The operational inactivation rate constant (k_{iop}) of immobilized enzyme at 35°C, with 10 m*M* urea in phosphate buffer (0.1 *M* m*M*, pH 7.2) was calculated to be 5.83×10^{-5} min^{-1}. Thus, the high operational stability obtained with urease immobilized onto pHEMA membranes indicate that this immobilized enzyme can successfully be used for continuous decomposition of urea from biological fluids.

Thermal Stability of Free and Immobilized Urease

Thermal stability experiments were carried out with the free and the immobilized enzymes, which were incubated in the absence of substrate at various temperatures. Figures 8 and 9 show

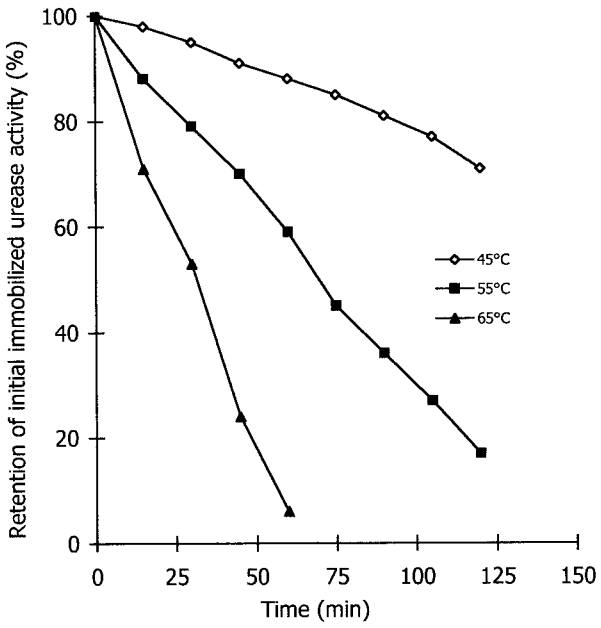

Figure 9 Influence of temperature on the stability of immobilized urease.

Table II Half-Lives ($t_{1/2}$) and Inactivation Rate Constants (k_i) of the Free and the Immobilized Urease at Three Different Temperature

	Free Enzyme		Immobilized Enzyme	
Temperature (°C)	$t_{1/2}$ (min)	k_i (10^3)	$t_{1/2}$ (min)	k_i (10^3)
45	130	4.38	250	2.13
55	53	13.75	73	8.80
65	19	36.33	31	21.13

the heat inactivation curves between 45–65°C for the free and the immobilized urease, respectively. At 45°C the free and the immobilized urease retained their activity to a level of 51 and 71% during a 120-min incubation period. At 55°C, the immobilized form was inactivated at a much slower rate than the native form. Both the free and the immobilized enzymes lost of all their initial activity at 65°C after a 60- and 75-min treatment, respectively. The half-live values and thermal inactivation rate constants for the free and the immobilized enzyme were determined from the percent residual activity vs. time, at three different temperatures, and presented in Table II. The half-live at 45°C was 130 min for the free enzyme and 250 min for the immobilized enzyme. The thermal inactivation rate constants (k_i) for free and immobilized urease at 45°C were found to be 4.38×10^{-3} and 2.13×10^{-3} min^{-1}, respectively. These results suggest that the thermostability of immobilized urease increased considerably as a result of covalent immobilization onto pHEMA membrane (Table II). The activity of the immobilized preparation, especially in a covalently bound system, is more resistant than that of the soluble form against heat and denaturing agents.[31] If the thermal stability of an enzyme were enhanced by immobilization, the potential utilization of such enzymes would be extensive. In principle, the thermal stability of an immobilized enzyme can be enhanced, diminished, or unchanged relative to free counterparts, and several examples for each kind have been previously reported.[9,13,25,30,32]

CONCLUSIONS

In this study, the pHEMA hydrogel in membrane form was prepared and used as support for the immobilization of model enzyme urease. The pHEMA membrane has high mechanical strength and high stability to many chemicals and microbial degradation. It is nontoxic and biocompatible. As previously mentioned, the optimum pH and temperature profile of the immobilized enzyme is not drastically modified, and the thermal stability of the urease was increased upon immobilization. The enzyme membrane was used continuously for urea decomposition with a 13% initial activity loss in packed bed reactor. The pHEMA in the membrane form elicit the desired properties, and could be used in covalently bound bioactive macromolecule immobilization. As presented in this model work, the enzyme membrane system could be also used in construction of a biosensor for determination of urea from biological fluids or especially in artificial kidney devices for the removal of urea from blood.

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