Retention of Activity of Urease Immobilized on Grafted Polymer Films

Kazunori Yamada, Yoshinori Iizawa, Jun-ichi Yamada, Mitsuo Hirata

Department of Applied Molecular Chemistry, College of Industrial Technology, Nihon University, Narashino, Chiba 275-8575, Japan

Received 6 December 2005; accepted 13 May 2006
DOI 10.1002/app.24861
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Expanded poly(tetrafluoroethylene) (ePTFE) films grafted with 2-hydroxyethyl methacrylate (HEMA) and 2-hydroxyethyl acrylate (HEA) were applied to a polymer support for urease immobilization. The HEMA- and HEA-grafted ePTFE (ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA) films prepared by the combined use of the plasma treatment and photografting possessed high water-absorptivities. Imidazole groups were introduced to grafted PHEMA and PHEA chains with 1,1′-carbonyldiimidazole (CDI) in acetonitrile. The activity of urease covalently immobilized to the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films in a pH 7.0 buffer at 4°C had the maximum value at the optimum pH value of 7.5 for native urease. Urease immobilized on the ePTFE-*g*-PHEMA films with the extent of CDI bonding of about 20% had the maximum activity, and the repeatedly measured activity was kept almost constant. The relative activity of immobilized urease stayed almost constant in the range of the immobilized amounts between 10 and 30 mg/g for both grafted ePTFE films, and decreased at higher immobilized amounts because of the crowding of immobilized urease molecules in the grafted layers. The relative activity of immobilized urease had the maximum values at the grafted amounts of 1.2 and 1.7 mmol/g for the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films, respectively, and the further increase in the grafted amount resulted in the decrease in the relative activity. The optimum temperature of the activity for immobilized urease was shifted from 30 to 50°C for native urease by the covalent immobilization on both grafted ePTFE films and immobilized urease was repeatedly usable without a considerable decrease in the activity in the regions of the pH 6.0–9.0 and 10–60°C. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 4886–4896, 2006

Key words: photografting; poly(tetrafluoroethylene); 2-hydroxyethyl metharylate; 2-hydroxyethyl acrylate; covalent immobilization; urease

INTRODUCTION

Urea has an important role as a fertilizer, which satisfies the nitrogen requirement of the plant. On the other hand, urease (urea aminohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea to form ammonia and carbon dioxide.[1,2] Urea is recognized as a polluting agent of agricultural sewage, and further application of urease has been extensively investigated in blood detoxification and in removal of urea from beverages and food. In fact, the most effective way of removing urea from aqueous solutions is the utilization of immobilized urease because no efficient adsorbent is available for urea.[3–9] However, the use of enzymes is often limited due to instability and the impossibility of separating them from the reaction medium after the catalytic process. To overcome these difficulties, many researchers have immobilized enzymes on different water-insoluble matrices.[10,11] Enzymes are immobilized on various insoluble matrices by physical or chemical methods. The physical methods are based mainly on enzyme adsorption or enzyme entrapment in the network structure.[12,13] Although these methods are very readily carried out, enzymes immobilized tend to be gradually released from the matrices. On the other hand, one of the chemical methods is formation of one or more covalent bonds between the amino acid residues of the enzymes and the reactive functional groups of the insoluble matrices. The chemical methods are effective in the fact that the covalent bonds are generally irreversible and the immobilized enzymes are not isolated.[1,3,4]

Many studies have been reported on immobilization of urease and the retention of the activity of immobilized urease.[3–9] However, most of these studies are concerned with the dependence of the activity of immobilized urease on the pH value, temperature, and immobilized amount; little was reported on the influence of the immobilization conditions such as the pH value on immobilization, the hydrophilic/hydrophobic balance, and the active site density of the support surfaces for urease immobilization. In

Correspondence to: K. Yamada (k5yamada@cit.nihon-u.ac.jp).

Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan.

fact, urease is immobilized at the same pH value as the optimum pH value of the activity of native urease, as published in most of the articles[3,5–9] In addition, polymer materials have been extensively used as the support matrices for urease immobilization, because they can be readily modified and have various functional groups.[14,15] Immobilization of enzymes on membranes or film surfaces offers some advantages over beaded supports.

To be suitable for the covalent binding of enzymes, the supports should have these properties: a hydrophilic nature, an appropriate density of chemically active site, resistance to biodegradation, and chemical and mechanical stability. In addition, the covalent binding of enzymes must be carried out under mild conditions to avoid their inactivation. Many researchers have reported on immobilization of enzymes on polymer matrices prepared by grafting hydrophilic or reactive monomers such as methacrylic acid (MAA),[16] acrylic acid (AA),[16–23] 2-hydroxyethyl methacrylate (HEMA),[9,24,25] glycidyl methacrylate (GMA),[26] and butyl methacrylate.[27] By careful selection of polymer support and the monomer, it is possible to control the hydrophilic/hydrophobic nature of the support surfaces and the density of chemically active site for enzyme immobilization.

We chose 2-hydroxyethyl methacrylate (HEMA) and 2-hydroxyethyl acrylate (HEA)-grafted expanded poly (tetrafluoroethylene) (ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA) films as support materials for urease immobilization because the ePTFE films possess good chemical stability and mechanical strength, PHEMA chains possess the biocompatibility, and the grafted layers formed have enough hydrophilicity to immobilize urease. In this study, the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were prepared by the combined use of the plasma treatment and photografting, and then urease was covalently immobilized onto the grafted PHEMA and PHEA chains activated with 1,1′-carbonyldiimidazole (CDI). Attempts were made to optimize coupling conditions as a function of the pH value on covalent immobilization, the grafted amount, and the immobilized amount. The activity of immobilized urease was measured at different pH values, and temperatures and their reusability was also estimated.

EXPERIMENTAL

Materials

An ePTFE film of diameter of 47 mm (thickness: 35 μm, pore size: 3.0 μm, degree of vacancy: 79%) from Toyo Roshi (Tokyo, Japan) was used as a polymer substrate. The ePTFE films were washed with water, methanol, and acetone in turn, and then dried under reduced pressure. HEMA and HEA were selected as a grafting hydrophilic monomer, because both monomers were neutral and had a reactive alcoholic OH group suitable for covalent immobilization of urease.[5,24,25] In addition, PHEMA and PHEA are water-soluble, not susceptible to microbial attack, and possess the biocompatibility, and the grafted layers formed have enough hydrophilicity to retain the activity of urease. The chemicals were used without further purification. Urease (EC 3.5.1.5, Type IX powder from Jack Beans) with the specific activity of 82.8 U/mg-solid was purchased from Sigma Chemical.

Plasma treatment and photografting

The grafting of HEMA and HEA onto the ePTFE films was carried out by the combined use of the plasma treatment and photografting, according to the procedure described in our previous article.[28,29] A Shimadzu LCVD 20 type plasma polymerization apparatus was used for the plasma treatment of the ePTFE surfaces. The ePTFE films were fixed on the sample holder in a bell-jar with double-sided Scotch tape. Both surfaces of the ePTFE films were treated with oxygen plasmas generated at output of 200 W for 120 s under vacuum of 6.67 Pa (0.05 Torr), while the sample holder was rotated at 60 rpm. The plasma-treated ePTFE films were preserved in an atmosphere of oxygen for 30 min to form oxygen-containing functional groups.[28,30] A Riko rotary photochemical reactor RH400-10W was used to prepare the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films. The plasma-treated ePTFE films were dipped in a 50 cm^3 of acetone solution containing 0.25 g benzophenone (BP) as a photosensitizer (0.50 w/v %) for 1 min to coat their surfaces with BP. Subsequently, the ePTFE films were immersed in an aqueous 80 vol % methanol solution of HEMA or HEA at a monomer concentration of 1.0*M* in the Pyrex glass tubes. The ePTFE films immersed in the monomer solutions (60 cm^3) were irradiated with UV rays emitted from a 400 W high-pressure mercury lamp at 60°C.[16,31–33] The amounts of grafted HEMA and HEA were calculated from the weight increase of the ePTFE films in mmol/g.[28]

Membrane properties of the grafted films

Membrane properties of the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were estimated in the manner described in our previous articles.[28,33,34] The surface areas of the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were measured with a slide caliper. The area ratio was calculated from the sizes after the photografting and the original size of 17.3 cm^2. The amount of absorbed water was calculated from the weight

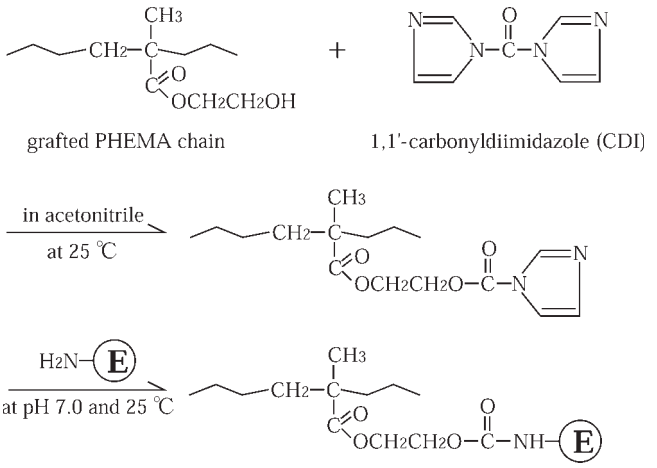

Figure 1 Schematic representation of immobilization of an enzyme on a grafted PHEMA chains through covalent bonding with 1,1-carbonyldiimidazole.

increase of the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films immersed in distilled water for 24 h at 30°C. In addition, n_{water} value, the number of bonded or assigned per HEMA or HEA monomer segment,[28,31] was calculated from the grafted amount and the amount of absorbed water according to eq. (3) in Ref. 34.

Immobilization of urease

The ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films swollen in acetonitrile were immersed in an acetonitrile solution (40 cm^3) containing 0.15 g 1,1′-carbonyldiimidazole (CDI) with moderate stirring for 2 h at 25°C to introduce imidazole groups to the grafted PHEMA and PHEA chains (see Fig. 1). After the reaction, the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were washed sequentially with a decreasing amount of acetonitrile in water (100, 75, 50, and 25 vol % of acetonitrile) and finally with water.[35] The extent of bonded CDI was determined by measuring the absorbances of the aliquots diluted 200-fold with acetonitrile at 210 nm after the reaction.

The CDI-activated ePTFE-*g*-PHEMA and PTFE-*g*-PHEA films were immersed in a pH 7.0 phosphate buffer ($I = 0.01M$) containing urease ($C = 0.5$ mg/cm^3) for 24 h at 4°C with moderate stirring. The amount of urease immobilized on the ePTFE-*g*-PHEMA and PTFE-*g*-PHEA films was determined from the decrease in the absorbance at 207 nm after immobilization. The urease-immobilized ePTFE-*g*-PHEMA and ePTFE-*g*-PHA [urease-i-(ePTFE-*g*-PHEMA) and urease-i-(ePTFE-*g*-PHEA)] films were washed with the buffers of the same pH values as those used on the activity measurements and preserved in the buffers at 4°C.

Activity measurements

The urease-i-(ePTFE-*g*-PHEMA) and urease-i-(ePTFE-*g*-PHEA) films were immersed in the buffers of the pH values of 6.0–9.0 containing 40 μmol of urea ($C = 1.0$ μmol/cm^3) at 30°C to start the enzymatic reaction. The activities of free and immobilized urease were estimated by determining the specific initial velocity from the relationship of the amount of ammonia liberated from the urease-catalyzed hydrolysis of urea against the reaction time. The ammonia concentration was spectrophotometrically determined by the reaction of phenol with hypochlorite.[36–38] A 2.0 cm^3 of aqueous solution containing phenol (0.106*M*) and sodium nitroprusside dihydrate (0.168 m*M*) were added to the aliquots (0.1 cm^3) taken from the reaction solutions at regular time intervals to stop the enzymatic reaction. Subsequently, a 2.0 cm^3 of aqueous solution of sodium hydroxide (0.125*M*) and sodium hypochlorite (11.8 m*M*) was added to the solution. After the mixtures were shaken at 37°C for 20 min for color development, the absorbance was read at 635 nm. The relative activity was expressed as the ratio of the specific initial velocity of the immobilized urease to that of native urease.

RESULTS AND DISCUSSION

Grafting of HEMA and HEA onto ePTFE Films

First, the grafting conditions were investigated for preparing ePTFE-*g*-PHEMA films and ePTFE-*g*-PHEA films with high grafted amounts. In our previous articles, water was used as a solvent for preparing solutions of water-soluble hydrophilic monomers.[31–33,39] In this study, an aqueous ethanol solution of 20 vol % was used in place of water as a solvent to increase the solubility of PHEMA and PHEA in the medium. Table I shows the results of the grafting of HEMA onto the ePTFE films under different conditions. PTFE-*g*-PHEMA films with higher grafted amounts were prepared by irradiating the UV rays onto the

TABLE I
Grafting of HEMA onto the pPTFE Films under Different Conditions

Plasma treatment[a]	BP coating[b]	UV irradiation[c]	Grafted amount (mmol/g)
Yes	Yes	Yes	18.90
No	Yes	Yes	0.42
Yes	Yes	No	0.092[d]
Yes	No	Yes	4.87
No	No	Yes	0.62

[a] Plasma treatment for 120 s at 200 W.
[b] Immobilization in a BP acetone solution of 0.0275 m*M*.
[c] Photografting at 60°C for 2.5 h.
[d] Thermal grafting at 60°C without UV irradiation.

plasma-treated and BP-coated ePTFE films immersed in a HEMA monomer solution. It can be considered that oxygen-containing functional groups formed on the ePTFE surfaces by the plasma treatment are effectively decomposed by the UV irradiation to act as an active site for photografting.[28,40,41]

On the other hand, the grafting of HEMA occurred little for the thermal grafting at 60°C without the UV irradiation, even though the ePTFE films were plasma-treated prior to the grafting. This means that the plasma treatment and the subsequent UV irradiation are required to effectively initiate the grafting of HEMA onto the ePTFE films and the BP coating is an additional procedure to further increase the grafted amount. Therefore, the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were prepared by the combined use of the plasma treatment and photografting, and the amounts of grafted HEMA and HEA were adjusted by varying the UV irradiation time. The amounts of grafted HEMA and HEA increased up to 18 mmol/g at 150 min and up to 4.5 mmol/g at 100 min, respectively. Our previous article showed that the grafted amounts of methacrylic monomers were higher than those of acrylic monomers in such a way that MAA > AA and MAAm > AAm.[31] Here, the amount of grafted HEMA was higher than the amount of grafted HEA at the same irradiation time.

Hydrophilization of the ePTFE films by the photografting of HEMA and HEA was estimated by the dimensional change and water-absorptivity. Figure 2 shows the changes the area ratio, amount of absorbed amount, and n_{water} value with the grafted amount for the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films. The ePTFE films was contracted with an increase in the amounts of grafted HEMA and HEA. The area ratio stayed almost constant in the range of the grafted amounts higher than 8 mmol/g for the ePTFE-*g*-PHEMA films and 2.5 mmol/g for the ePTFE-*g*-PHEA films. The contraction of the ePTFE films is considered to be mainly due to hydrogen bonding between hydroxyl groups appended to grafted PHEMA and PHEA chains. Since a HEA monomer has no α-methyl group and is structurally more hydrophilic than a HEMA monomer, the location of the photografting of HEA is considered to be restricted to the outer surface region of the ePTFE film. Therefore, the ePTFE-*g*-PHEA films were more highly contracted than the ePTFE-*g*-PHEMA films.

The amount of absorbed water increased with an increase in the grafted amount for both grafted ePTFE films and the ePTFE-*g*-PHEA films had higher water-absorptivity than the ePTFE-*g*-PHEMA films. In addition, the water-absorptivity of the grafted layers formed was estimated from the n_{water} value. The n_{water} value for both grafted ePTFE films sharply decreased against the grafted amounts, and then stayed almost constant in the range of the grafted amounts where the constant area ratio was obtained in Figure 2(a).

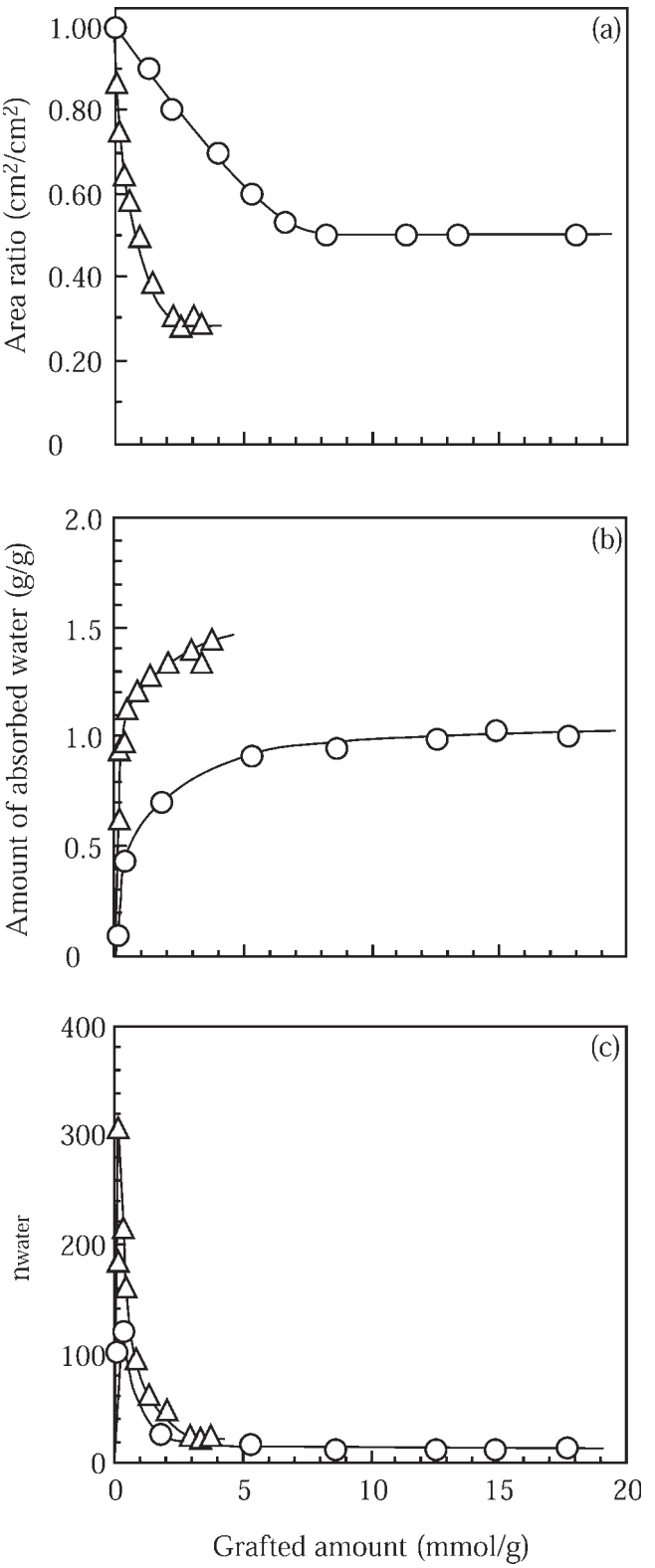

Figure 2 Changes in (a) the area ratio, (b) amount of absorbed water, and (c) n_{water} value with the grafted amount for the ePTFE-*g*-PHEMA (○) and ePTFE-*g*-PHEA (△) films.

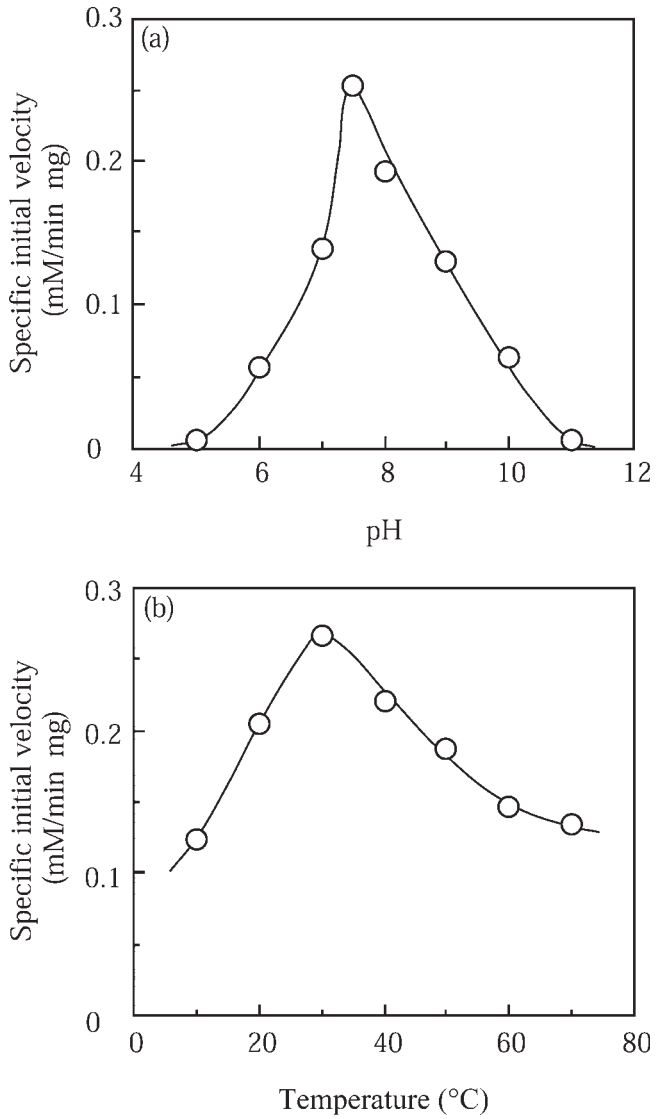

Figure 3 Variations in the specific initial velocity with (a) the pH value at 30°C and (b) the temperature at pH 7.5 for native urease. Urease concentration: 0.5 μg/cm^3. Urea concentration: 0.5 m*M*.

Activity of native urease

The optimum temperature and pH value of the activity of an enzyme are slightly different from one article to another.[5–9] Therefore, the optimum pH value and temperature of the activity of urease used in this study were determined. The pH dependence of the activity of native urease (0.5 μg/cm^3) was estimated in a urea solution of 1.0 m*M* at 30°C As shown in Figure 3(a), the specific initial velocity of native urease had the maximum value at pH 7.5. Subsequently, the temperature dependence of the activity of native urease was estimated at pH 7.5. It was found from Figure 3(b) that the specific initial velocity of native urease used had the maximum value at 30°C. Therefore, the specific initial velocity of urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films was estimated at pH 7.5 and 30°C as the optimum condition for native urease used. The initial velocity determined from the linear relationship between the reaction time and the concentration of ammonium ions generated by the enzymatic reaction in the initial reaction state was directly proportional to the urease concentration in the urease concentration range below 15 μg/cm^3. The specific initial velocity for native urease at pH 7.5 and 30°C was calculated to be 0.30 m*M*/(mg min) from the slope of the linear relationship.

Immobilization of urease onto ePTFE-*g*-PHEMA films

The reaction time dependence of the degrees of CDI bonding was investigated at 25°C for the ePTFE-*g*-PHEMA films with the grafted amounts of 1.31, 5.37, and 12.52 mmol/g.[35] Figure 4 shows the changes in the extent of CDI bonding with the reaction time for the ePTFE-*g*-PHEMA films of three different grafted amounts. The extent of CDI bonding increased with an increase in the reaction time irrespective of the grafted amount. The extent of CDI bonding went up to 95% at 120 min for an ePTFE-*g*-PHEMA film with the grafted amount of 1.31 mmol/g, and increased with a decrease in the amount of grafted HEMA at the same reaction time. This is why the ePTFE films highly contracted by the photografting of HEMA and the denser grafted layers were formed at higher grafted amounts as shown in Figure 2(a). Native urease of the same amount as urease immobilized on

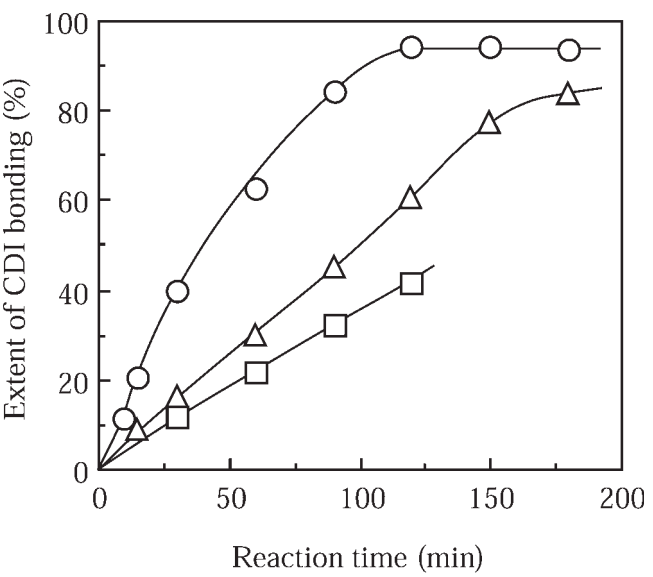

Figure 4 Changes in the extent of CDI bonding with the reaction time for the ePTFE-*g*-PHEMA films with the grafted amounts of 1.31 (○), 5.37 (△), and 12.52 (□) mmol/g at 25°C. CDI concentration: 3.75 × 10^{-3} g/cm^3.

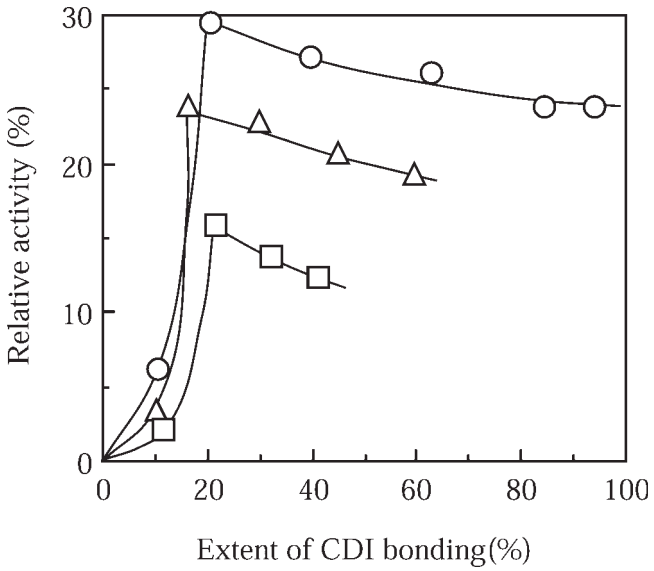

Figure 5 Variations in the relative activity with the extent of CDI bonding for urease immobilized on the ePTFE-*g*-PHEMA films with the grafted amounts of 1.31 (○), 5.37 (△), and 12.52 (□) mmol/g at pH 7.5 and 30°C. Urea concentration: 0.5 m*M*.

each ePTFE-*g*-PHEMA film was dissolved in a pH 7.5 buffer of 40 cm^3, and then the specific initial velocity of native urease was determined at 30°C. The relative activity of immobilized urease was obtained from the ratio of the specific initial velocity of urease immobilized on the ePTFE-*g*-PHEMA films to that of native urease.

Figure 5 shows the variations in the relative activity with the extent of CDI bonding for urease immobilized on ePTFE-*g*-PHEMA films of different grafted amounts at pH 7.5 and 30°C. The relative activity had the maximum values at the extent of CDI bonding of about 20% and then gradually decreased with an increase in the extent of CDI bonding irrespective of the grafted amount. Subsequently, the activity was repeatedly measured at different temperatures for urease immobilized on the ePTFE-*g*-PHEMA films of the grafted amount of 5.37 mmol/g with the extents of CDI bonding of 16.8 and 45.0%.

As shown in Figure 6, the specific initial velocity for urease immobilized on the ePTFE-*g*-PHEMA film with the extent of CDI bonding of 16.8% stayed constant in the temperature range below 50°C, but gradually decreased against the run number of the activity measurements at 60°C. After the activity measurements were repeatedly measured five times at 60°C, the activity measurements at 10°C were carried out. The activity at 10°C measured after the activity measurements at 60°C was much lower than that directly measured at 10°C. On the other hand, the activity for urease immobilized on the ePTFE-*g*-PHEMA film with the extent of CDI bonding of 45.0% stayed constant even at 60°C and the activity at 10°C measured after the activity measurement at 60°C was as high as that directly measured at 10°C. These results suggest that urease molecules are immobilized on the ePTFE-*g*-PHEMA films by the multipoint attachment through covalent bonding between CDI groups attached to the grafted PHEMA chains and amine groups of urease molecules as shown in Figure 1. In particular, a urease would be highly immobilized on the ePTFE-*g*-PHEMA films in the range of the extents of CDI bonding above 20%. This will lead the constant activity at 60°C and prevent immobilized urease molecules to undergo thermal denaturation at higher temperature.[16,23,42] However, when the number of the covalent bonds between a urease molecule and grafted PHEMA chains are further increased, the activity of immobilized urease decreased probably

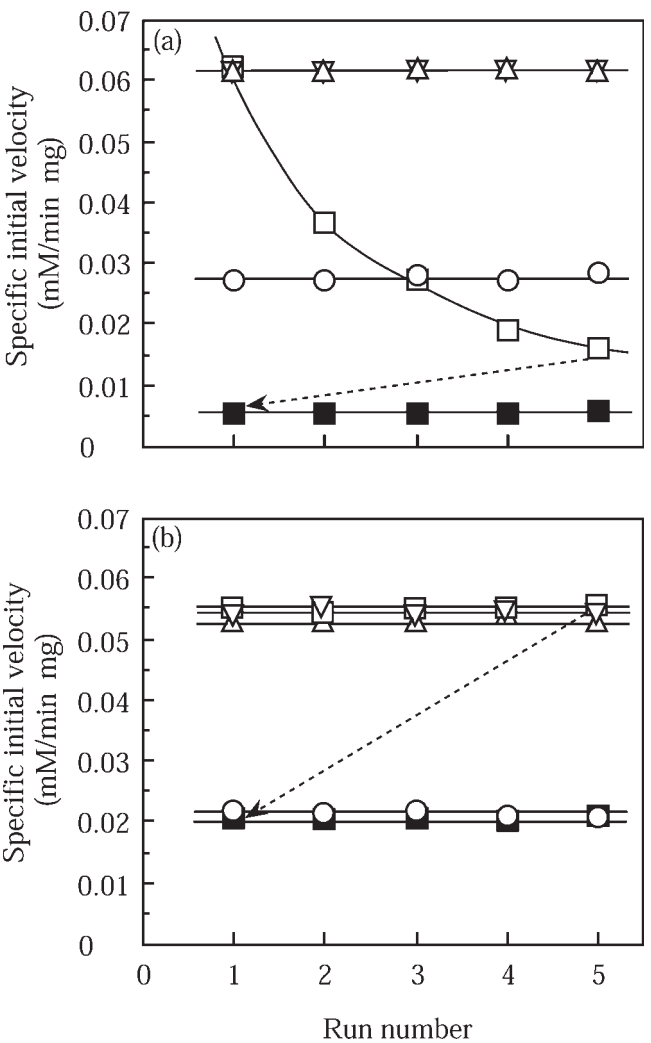

Figure 6 Reusability of urease immobilized on the ePTFE-*g*-PHEMA films of 5.37 mmol/g with the extents of CDI bonding of (a) 16.8 and (b) 45.0% at 10 (○), 30 (△), 50 (△), 60°C (□), and 10°C after the activity measurements at 60°C (■) in a pH 7.5 buffer. Immobilized amount: (a) 21.8 mg/g, (b) 23.0 mg/g.

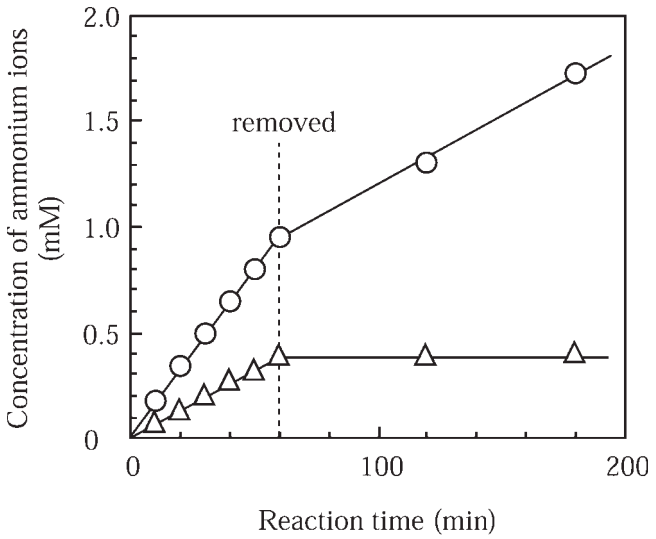

Figure 7 Changes in the concentration of ammonium ions with the reaction time for urease immobilized on the ePTFE-*g*-PHEMA films of 5.32 mmol/g with the extent of CDI bonding of 7.2 (○) and 59.0 (△)% at pH 7.5 and 30°C. The urease-i-(ePTFE-*g*-PHEMA) films were removed from the urea solutions at 60 min.

due to conformational changes. In addition, urease immobilized on the ePTFE-*g*-PHEMA films with lower grafted amounts had higher relative activity. As urease was also immobilized on grafted PHEMA chains in the inside of the grafted layer at higher grafted amount, diffusion of urea molecules into the grafted layers was suppressed, resulting in a decrease in the relative activity.[19,22,43]

Consequently, release of urease molecules from the ePTFE-*g*-PHEMA films with different extents of CDI bonding was followed up during the activity measurements. The amounts of urease immobilized on the ePTFE-*g*-PHEMA films of 5.32 mmol/g with the extents of CDI bonding of 7.2 and 59.0% were 18.7 and 7.4 mg/g, respectively. Figure 7 shows the changes in the concentration of ammonium ions generated by urease immobilized on these two ePTFE-*g*-PHEMA films with the reaction time at pH 7.5 and 30°C. The concentration of ammonium ions enzymatically generated increased linearly against the reaction time for both urease-immobilized ePTFE-*g*-PHEMA films. When urease-i-(ePTFE-*g*-PHEMA) film of the extent of CDI bonding of 59.0% was removed from the urea solution, the increase in the concentration of ammonium ions stopped. This indicates that most of the urease molecules physically adsorbed on the ePTFE-*g*-PHEMA film are removed prior to the activity measurement and no urease molecules covalently immobilized on the ePTFE-*g*-PHEMA film are liberated during the activity measurement. On the other hand, for the urease-i-(ePTFE-*g*-PHEMA) film of the extent of CDI bonding of 7.2% the concentration of ammonium ions gradually increased even after it was taken out of the urea solution. These results support that some urease molecules physically adsorbed were released from the ePTFE-*g*-PHEMA film during the activity measurement.

Activity of immobilized urease

Effect of pH value on immobilization

The activity of immobilized urease was measured as a function of the pH value on immobilization, the amounts of grafted HEMA and HEA, the amount of immobilized urease, and the pH value and temperature at the activity measurements.

First, the effect of the pH value on immobilization on the activity of immobilized urease was examined. Many studies have been carried out on the dependence of the activity of covalently immobilized enzymes on the pH value and temperature at the activity measurements.[22,23,25,42,44] However, very few articles have been reported on the effect of the pH value on immobilization on the activity of immobilized enzymes.[16,22] In most of the articles referred earlier, enzymes were covalently immobilized with different coupling agents at the pH values equal to the optimum pH values of each enzymatic activity. We reported in our previous article[16] that the activity of trypsin covalently immobilized on the AA-grafted PE plates at pH 6.0 with a water-soluble carbodiimide was much higher than that of trypsin immobilized at 7.8 as the optimum pH value of the activity of native trypsin. Therefore, in this study, we began with the determination of the pH value on immobilization. The activity of urease immobilized on an ePTFE-*g*-PHEMA film with the grafted amount of 5.35 mmol/g and an ePTFE-*g*-PHEA film with 1.41 mmol/g in the pH range from 6.0 to 9.0 was measured at pH 7.5 and 30°C as the optimum conditions for native urease as determined in Figure 3. Figure 8 shows the variations of the relative activity of urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films with the pH value on immobilization. Urease immobilized at pH 7.0 had the maximum activity values for both grafted ePTFE films. This pH value was a little lower than the optimum pH value of 7.5 for the activity of native urease. This pH difference is considered to arise from the change in the isoelectric point due to a decrease in the amino groups of a urease molecule through the covalent reaction, although it is difficult to make more detailed discussions here.

Effect of pH value on the activity measurement

The activity of urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films at pH 7.0 was measured in buffers of different pH values at 30°C.

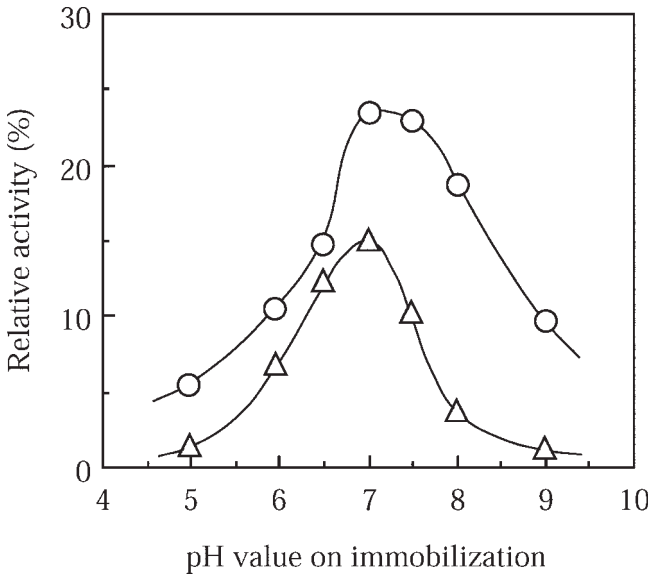

Figure 8 Variations in the relative activity of urease immobilized on an ePTFE-*g*-PHEMA film of 5.36 mmol/g (○) and an ePTFE-*g*-PHEA film of 1.75 mmol/g (△) with the pH value on immobilization at pH 7.5 and 30°C. Average immobilized amount: 15–22 mg/g for an ePTFE-*g*-PHEMA film, 12–17 mg/g for an ePTFE-*g*-PHEA film.

Figure 9 shows the variations in the relative activity of urease immobilized on the ePTFE-*g*-PHEMA (G = 5.64 mmol/g) and ePTFE-*g*-PHEA (G = 1.41 mmol/g) films with the pH value on the activity measurement. The activity of urease immobilized on both grafted ePTFE films had the maximum values at pH 7.5 as the optimum pH value of native urease. Therefore, the effects of the grafted amount and the immobilized amount on the activity of urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films at pH 7.0 will be examined at pH 7.5 in the following sections.

Effect of grafted amounts

It has been generally known that many enzymes exhibit higher activity in the case where they are immobilized on more hydrophilic insoluble supports.[16] Therefore, the activity of urease immobilized on the ePTFE-*g*-PHEA films was also measured as a function of the grafted amount. Urease was immobilized on ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films with different grafted amounts to prepare the urease-immobilized samples with an almost constant immobilized amount (the average immobilized amounts: 23.9 mg/g for the ePTFE-*g*-PHEMA films and 11.3 mg/g for the ePTFE-*g*-PHEA films), and then the activity of each immobilized urease was measured at pH 7.5 and 30°C. Figure 10 shows the variations in the relative activity with the grafted amounts for the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films. The activity of immobilized urease had the maximum values at the grafted amounts of 1.2 and 1.7 mmol/g for the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films, respectively, and then decreased with an increase in the grafted amount. The grafted layers formed becomes thicker with the increase in the grafted

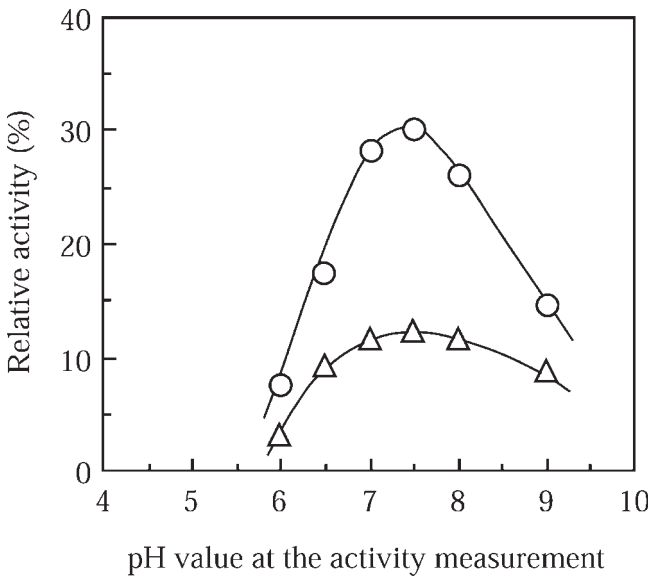

Figure 9 Variations in the relative activity of urease immobilized on an ePTFE-*g*-PHEMA film of 5.64 mmol/g (○) and an ePTFE-*g*-PHEA film of 1.41 mmol/g (△) with the pH value at the activity measurement at 30°C. Average immobilized amount: 9.5 mg/g for an ePTFE-*g*-PHEMA film, 10–13 mg/g for an ePTFE-*g*-PHEA film.

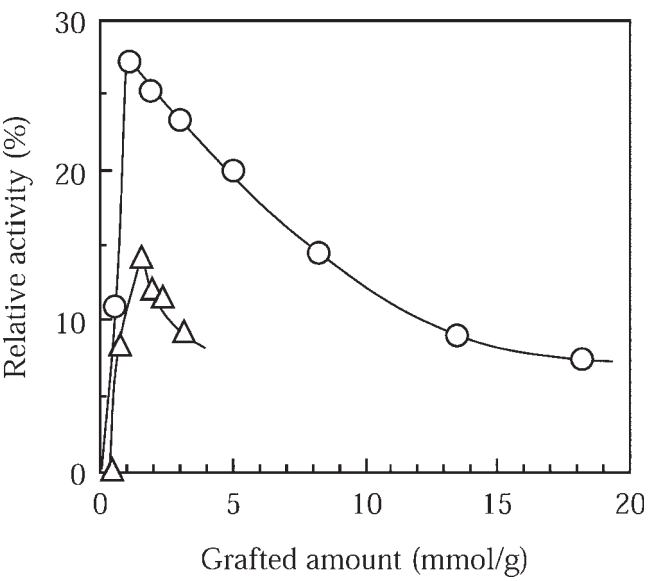

Figure 10 Changes in the relative activity of urease immobilized on the ePTFE-*g*-PHEMA (○) and PTFE-*g*-PHEA (△) films with the grafted amount at pH 7.5 and 30°C. Average immobilized amount: 23.9 mg/g for an ePTFE-*g*-PHEMA film, 11.3 mg/g for an ePTFE-*g*-PHEA film.

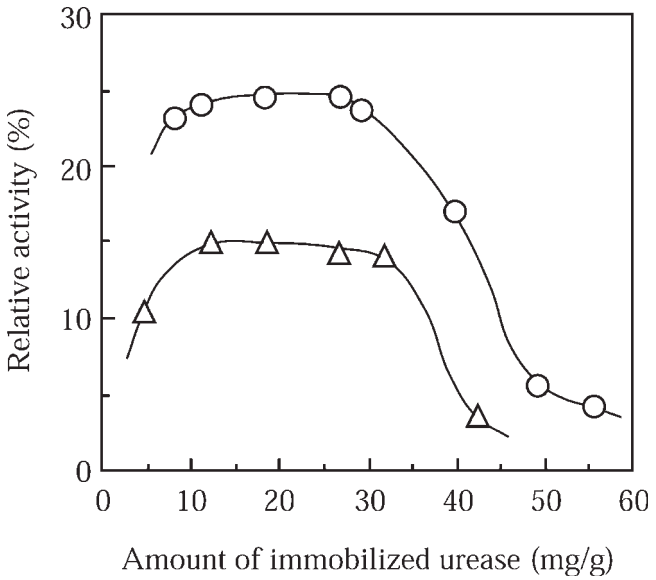

Figure 11 Variations in the relative activity of urease immobilized on the ePTFE-*g*-PHEMA (2.68 mmol/g) (○) and PTFE-*g*-PHEA (1.42 mmol/g) (△) films with the immobilized amount at pH 7.5 and 30°C. Average immobilized amount: 23.9 mg/g for an ePTFE-*g*-PHEMA film, 11.3 mg/g for an ePTFE-*g*-PHEA film.

amount. However, the contraction of the ePTFE films by photografting results in the formation of the dense grafted layers. Since urease is also immobilized on grafted PHEMA and PHEA chains in the inside of the grafted layer at higher grafted amounts, the diffusion of urea molecules in the grafted layers would get depressed, resulting in a decrease in the relative activity. However, contrary to our expectations, the maximum activity value obtained for urease immobilized on the ePTFE-*g*-PHEA films was a little lower than that obtained for the ePTFE-*g*-PHEMA films. Since the ePTFE-*g*-PHEA films prepared in this study had relatively low grafted amounts, the hydrophobicity of ePTFE is considered to have some advertise influences on the activity of the immobilized urease.

Effect of immobilized amounts

The effect of the amount of immobilized urease on the activity of urease immobilized on an ePTFE-*g*-PHEMA film of 2.68 mmol/g and an ePTFE-*g*-PHEA film of 1.42 mmol/g was examined at pH 7.5 and 30°C. Figure 11 shows the variations in the relative activity with the immobilized amount for the urease-i-(ePTFE-*g*-PHEMA) and urease-i-(ePTFE-*g*-PHEA) films. The relative activity of immobilized urease stayed almost constant in the range of the grafted amounts from 10 to 30 mg/g for both grafted ePTFE films. However, further increase in the immobilized amount resulted in a sharp decrease in the activity. Here, the amount of native urease equivalent to urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films and 0.040 mmol of urea were dissolved in a pH 7.5 buffer of 40 cm^3, and the activity measurements were carried out at 30°C. Since native urease had a constant specific initial velocity of 0.30 m*M*/(mg min) as aforementioned, we can safely say that an increase in the immobilized amount leads to the crowding of urease molecules in the PHEMA layers, resulting in spatial restrictions, blocking of active sites, and/or denaturation of enzymes.[20,43] The urease-i-(ePTFE-*g*-PHEMA) and urease-i-(ePTFE-*g*-PHEA) films were washed with a pH 7.5 buffer after each activity measurement, and the activity was repeatedly measured. The specific initial velocity obtained at five activity measurements stayed almost constant. This indicates that no urease covalently immobilized was liberated from the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films during the activity measurements and storage.

Effect of temperature on the activity measurements

The thermal stability is one of the important factors in using the immobilized enzymes. The activity of urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films was repeatedly measured in the temperature range from 20 to 70°C at pH 7.5. Figure 12 shows the variation in the relative activity of immobilized urease with the reaction temperature. Here, the relative activity was determined from the

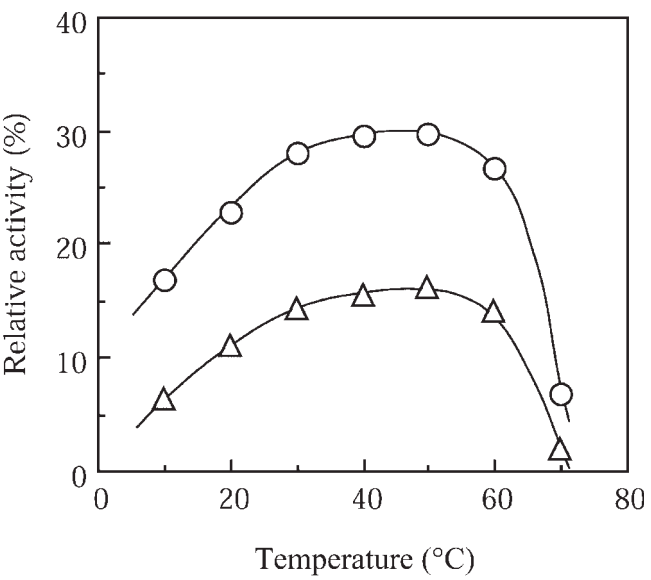

Figure 12 Variations in the relative activity of urease immobilized on the ePTFE-*g*-PHEMA (5.64 mmol/g) (○) and PTFE-*g*-PHEA (1.62 mmol/g) (△) films with the temperature at pH 7.5. Average immobilized amount: 8.6 mg/g for an ePTFE-*g*-PHEMA film, 11.5 mg/g for an ePTFE-*g*-PHEA film.

ratio of the specific initial velocity of immobilized urease at different temperatures to that of native urease at 30°C. The activity of native urease sharply decreased in the temperature range higher than 30°C as shown in Figure 3(b). On the other hand, the activity of immobilized urease gradually increased with an increase in the temperature range of 10–50°C, although the activity of immobilized urease was a little lower than that of native urease. In addition, the activity of immobilized urease stayed unchanged, even when the activity measurements were repeated at least five times in the temperature range below 60°C. However, the relative activity gradually decreased at 70°C by the repeated activity measurements because of further thermal denaturation.[16,23,42] It was made clear from the above results that an enhancement in the thermal stability can be attributed to the prevention of thermal denaturation probably due to the multipoint attachment between a urease molecule and grafted PHEMA or PHEA chains.

CONCLUSIONS

The ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were prepared by the combined use of the plasma treatment and photografting, and grafted polymer chains of the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films were applied to the covalent immobilization of urease. Imidazole groups were introduced to grafted PHEMA and PHEA chains with CDI in acetonitrile, and then urease was covalently immobilized to the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films at pH 7.0. The activity of urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films had the maximum value at the optimum pH value of 7.5 for native urease. The relative activity of immobilized urease stayed almost constant in the range of the immobilized amounts between 10 and 30 mg/g for both grafted ePTFE films, and decreased probably because of the crowding of immobilized urease molecules in the grafted layers at further increased immobilized amounts. The relative activity of immobilized urease had the maximum values at the grafted amounts of 1.2 and 1.7 mmol/g for the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films, respectively, and further increase in the grafted amount resulted in the decrease in the relative activity. The optimum temperature of the activity for immobilized urease was shifted from 30 to 50°C for native urease by the covalent immobilization on the both grafted ePTFE films and immobilized urease was repeatedly usable without a considerable decrease in the activity in the regions of the pH 6.0–9.0 and 10–60°C. In this study, the optimum conditions mentioned earlier, such as the pH values on immobilization and at the activity measurements, the grafted amounts, and the immobilized amount were determined in immobilizing urease on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films. The experimental results made clear that the thermal stability of urease can be improved by covalent coupling of urease on the ePTFE-*g*-PHEMA films, and urease immobilized on the ePTFE-*g*-PHEMA and ePTFE-*g*-PHEA films can be repeatedly used without a considerable decrease.

References

1. Chen, J. P.; Chiu, S. H. Enzyme Microb Technol 2000, 26, 359.
2. Rajesh; Bisht, V.; Takashima, W.; Kaneko, K. Biomaterials 2005, 26, 3683.
3. Martino, S. D.; El-Sheriff, H.; Diano, N.; De Maio, A.; Grano, V.; Rossi, S.; Bencivenga, U.; Mattei, A.; Mita, D. G. Appl Catal B: Environ 2003, 46, 613.
4. Ayhan, F.; Ayhan, H.; Piskin, E.; Tanyolaç, A. Bioresour Technol 2002, 81, 131.
5. Srinivasa Rao, M.; Chellapandian, M.; Krishnan, R. R. V. Bioprocess Eng 1995, 13, 211.
6. Huang, T.-C.; Chen, D.-H. Chem Eng Commun 1993, 120, 191.
7. Lin, C.-C.; Yang, M.-C. Biomaterials 2003, 24, 1989.
8. Huang, T.-C.; Chen, D.-H. J Chem Eng Jpn 1992, 25, 458.
9. Miyata, T.; Jikihara, A.; Nakamae, K. J Appl Polym Sci 1997, 63, 1579.
10. Chaney, A. L.; Marbach, E. P. Clin Chem 1962, 8, 130.
11. Aköl, S.; Yalçinkaya, Y.; Bayramoğlu, G.; Denizli, A.; Arica, M. Y. Process Biochem 2002, 38, 675.
12. Furuki, S. In Enzymes in Industry; Aehle, W., Ed.; Wiley-VCH: Weinheim, 2004; Section 3.3.
13. Scouten, W. H. In Methods in Enzymology, Vol. 135: Immobilized Enzymes and Cells; Mosbach, K., Ed.; Academic Press: New York, 1987, pp 30–65, .
14. Bulmus, V.; Ayhan, H.; Piskin, E. Chem Eng J 1997, 65, 71.
15. Yamada, K.; Nakasone, T.; Hirata, M. Trans Mater Res Soc Jpn 2001, 26, 1331.
16. Yamada, K.; Nakasone, T.; Nagano, R.; Hirata, M. J Appl Polym Sci 2003, 89, 2003.
17. Hsiue, G.-H.; Wang, C.-C.; Chen, C.-Y.; Chang, C.-J. Angew Makromol Chem 1990, 179, 149.
18. Abdel-Hey, F. I.; Beddows, C. G.; Gil, M. H.; Guthrie, J. T. J Polym Sci Polym Chem Ed 1983, 21, 1983.
19. Turmanova, S.; Trifonov, A.; Kalaijiev, O.; Kostov, G. J Membr Sci 1997, 127, 1.
20. Kulic, E. A.; Kato, K.; Ivanchenko, M. I.; Ikada, Y. Biomaterials 1993, 14, 763.
21. Li, Z. F.; Kang, E. T.; Neoh, K. G.; Tan, K. L. Biomaterials 1998, 19, 45.
22. Shimomura, M.; Ohta, M.; Sugiyama, N.; Oshima, K.; Yamauchi, T.; Miyauchi, S. Polym J 1999, 31, 274.
23. Wnag, C.-C.; Hsuiue, G.-H. J Appl Polym Sci 1993, 50, 1141.
24. Alves Da Silva, M.; Gil, M. H.; Guiomar, A. J.; Martins, C. J Appl Polym Sci 1990, 41, 1629.
25. Pashova, V. S.; Georgiev, G. S.; Dakov, V. A. J Appl Polym Sci 1994, 51, 807.
26. Portaccio, M.; El-Masry, M.; Diano, N. R.; De Maio, A.; Grano, V.; Lepore, M.; Travascio, P.; Bencivenga, U.; Pagliuca, N.; Mita, D. G. J Mol Catal B: Enzymatic 2002, 18, 49.
27. El-Sherif, H.; Martelli, P. L.; Casadio, R.; Portaccio, M.; Bencivenga, U.; Mita, D. G. J Mol Catal B: Enzymatic 2001, 14, 15.
28. Yamada, K.; Ebihara, T.; Gondo, T.; Sakasegawa, S.; Hirata, M. J Appl Polym Sci 1996, 61, 1899.
29. Yamada, K.; Hayashi, K.; Sakasegawa, K.; Onodera, H.; Hirata, M. Nippon Kagaku Kaishi 1994, 1994, 427 (in Japanese).

30. Yamada, K.; Kimura, J.; Hirata, M. J Photopolym Sci Technol 1998, 11, 263.
31. Yamada, K.; Tsutaya, H.; Tatekawa, S.; Hirata, M. J Appl Polym Sci 1992, 46, 1065.
32. Yamada, K.; Kimura, J.; Hirata, M. J Appl Polym Sci 2003, 87, 2244.
33. Yamada, K.; Shibuya, M.; Takagi, C.; Hirata, M. J Appl Polym Sci 2006, 99, 381.
34. Yamada, K.; Tatekawa, S.; Hirata, M. J Colloid Interface Sci 1994, 164, 144.
35. Zhuang, P.; Butterfield, D. A. J Appl Polym Sci 1993, 47, 1329.
36. Weatherburn, M. W. Anal Chem 1967, 39, 971.
37. Chen, J. P.; Chiu, S. H. Bioprocess Eng 1999, 21, 323.
38. Bolleter, W. T.; Bushman, C. J.; Tidwell, P. W. Anal Chem 1961, 33, 592.
39. Yamada, K.; Takeda, S.; Hirata, M. ACS Symp Ser 2003, 847, 511.
40. Osada, Y.; Iriyama, Y.; Thin Solid Films 1984, 118, 197.
41. Suzuki, Y.; Kishida, A.; Iwata, H.; Ikada, Y. Macromolecules 1986, 19, 1804.
42. Emi, S.; Murase, Y. J Appl Polym Sci 1990, 41, 2753.
43. Ganapathi, S.; Butterfield, D. A.; Bhattacharyya, D. J Chem Technol Biotechnol 1995, 64, 157.
44. Pariente, F.; Lorenzo, E.; Tobalina, F.; Abruña, H. D. Anal Chem 1995, 67, 3936.