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Acrylamide Grafted Poly(ethylene terephthalate) Fibers Activated by Glutaraldehyde as Support for Urease

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ABSTRACT

Urease was covalently immobilized on acrylamide-grafted poly (ethylene terephthalate) fibers after glutaraldehyde activation. Urease-containing fibers showed a very high operational stability and reusability, with about 85% of the initial activity after 90 d. The thermostability of the bound urease was positively influenced, and a slight change in optimum temperature was observed after immobilization, when compared with the free enzyme. The pH optimum of both types of urease was found to be the same, but immobilized urease showed an increased stability in a broader range of pH. The kinetic studies exhibited a slightly higher K_m value for the bound enzyme, with a value of 4.50 mmol dm^{-3}, when compared with the free enzyme (2.82 mmol dm^{-3}), which demonstrated that the immobilization procedure did not cause an unfavorable conformation for the substrate-product formation and a hindered diffusion. The graft yield was also found effective on maximum activity of immobilized urease. Twenty-five percent of the acrylamide-grafted fibers exhibited the highest enzymatic activity together with the highest water uptake. Higher graft yields were not suitable for the immobilization of the enzyme molecules as a result of crosslinks formed between the poly(acrylamide) chains and glutaraldehyde.

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Index Entries: Urease immobilization; poly(ethylene terephthalate) fibers; graft polymerization; acrylamide grafting; glutaraldehyde activation; urea hydrolysis.

Nomenclature: Aam, acrylamide; Bz_2O_2, benzoyl peroxide; -g-, grafted; GA, glutaraldehyde; PET, poly(ethylene terephthalate).

INTRODUCTION

Various methods of enzyme immobilization have been widely studied in the past and a large number of supports have been used according to the properties required in their application (*1–4*). Although grafting is a well-known method for the modification of polymer structures (*5,6*), it has rarely been used for immobilization purposes (*7–9*). Grafted polymers represent an advantageous group that might possess desired chemical and physical characteristics that will lead to a suitable microenvironment, influencing the properties of the immobilized species, according to their designation (*10*).

Poly(ethylene terephthalate) (PET) fiber is one of the most important synthetic fibers used in the textile industry today. Its undesirable features, such as low moisture regain, hydrophobic character, and lack of chemically-reactive groups, can be improved by grafting vinyl monomers onto PET fibers by using radiation or chemical methods (*11*). In an earlier study, we grafted acrylamide onto PET fibers by using benzoyl peroxide as the initiator (*12*), but the immobilization properties of acrylamide-grafted PET fibers are not readily known.

In the present study, urease enzyme, which catalyzes the hydrolysis of urea to ammonium and carbon dioxide, was coupled with acrylamide-grafted PET fiber by using glutaraldehyde. The most important uses of immobilized urease are in artificial kidney machines for blood detoxification and in industry for the removal of urea from foods and beverages (*2*). Attempts have been made to immobilize urease on toyopearl (*13*), clay minerals (*14*), activated silica support (*15*), poly(ethylenimine) cotton cloth (*16*), o-alkylated nylon tubes (*17*), and other supports. We have used poly(acrylamide)-gelatin gels (*2*), xanthan-alginate spheres (*18*), cross-linked gelatin (*19*), and carboxymethylcellulose (*20*) as support materials for the immobilization of urease in earlier studies, with somewhat successful results.

Here, the preparation and properties of urease-immobilized GA-activated Aam-grafted PET fibers have been described. The storage and operational conditions, together with pH and temperature characteristics, have been investigated. Effects of graft yield, GA, and enzyme concentration on maximum activity were determined. Water uptake of the fibers were tested and the kinetics of free and immobilized urease were also compared to understand how binding affected catalytic behavior.

MATERIALS AND METHODS

Urease from sword beans (5 U/mg; lyophilized) was obtained from Sigma (St. Louis, MO); PET fibers (multifilament, 99 denier) were supplied from Sasa (Adana, Turkey); and Bz_2O_2, acrylamide, urea, chemicals used to prepare reagents, buffer solutions, and the solvents were obtained from Merck (Darmstadt, Germany), which were in analytical pure grade. Bz_2O_2 was twice precipitated from chloroform solution in methanol and dried in a vacuum desiccator for 2 d. Benzene was crystallized by cooling in the refrigerator at 4°C, then crystallized part was used to prepare the Bz_2O_2 solutions.

Graft Polymerization

The fiber samples were washed with distilled water at 40°C, Soxhlet-extracted for 6 h with acetone and dried at ambient conditions before grafting. The fibers (0.1 ± 0.01 g samples) were placed in 45 mL deionized water containing 2 g of Aam. Then, 5 mL of Bz_2O_2 solution prepared in benzene (9.68 g/L) was added to initiate the reaction. The polymerization tube was put in a water bath (Kötterman, Hänigsen, Germany) at 75 ± 0.1°C. The grafted PET fibers were taken at specified time intervals (30–120 min) in order to obtain different graft yields (*12*), and subjected to a prewashing procedure with distilled water at 25°C for 2 h before being washed in boiling water for 4 h. The washing water was changed at least six times. The fibers were Soxhlet-extracted with water for 6 h to remove the homopolymer, which may remain as a film on the surface of the fibers, and subjected to a further washing procedure (2 h/25°C). Then they were dried and the graft yield (%) was calculated from the following equation:

$$\text{graft, \%} = w_2 - w_1 / w_1 \times 100$$

where w_1 and w_2 were original and grafted fiber weights, respectively.

Glutaraldehyde Activation and Immobilization

Acrylamide grafted PET fibers (1.0 ± 0.01 g, each) were soaked in 2.5 mL GA solution of varying concentrations (0.00128*M*–1.92*M*) for an hour at 25°C. Then the fibers were washed intensively for 30 min with redistilled water. This period was enough to remove the GA odor.

GA-activated grafted fibers were incubated for 24 h at 4°C in 2.5 mL urease solution (0.2 U or 0.04 mg/mL–3.2 U or 0.64 mg/mL), prepared using 0.1*M* phosphate buffer (pH 6.5) containing 0.1% (w/v) EDTA. Excess enzyme was washed from the fibers by redistilled water until no urease activity could be detected from the washing solutions. The immobilized urease was stored in double-distilled water at 4°C, until use.

Enzyme Assay

Bound urease activity was measured according to Weatherburn method (*21*) with slight modification: 2.1 mL of 0.1*M* phosphate buffer (pH 6.5) containing 0.1% (w/v) EDTA was added into test tubes containing 0.1 g urease immobilized hanked fiber. After preincubation for 2 min at 37°C, the enzymatic reaction was started by the addition of 0.3 mL urea solution (0.2 g/L) and vortexing. At the end of the assay (15 min), 1 mL of phenol (0.659*M*)-nitroprussiate (0.839 m*M*) and 1 mL of NaOH (0.5*M*)-NaOCl (0.35*M*) solutions were added to stop the reaction, after removing the hanked fiber sample from the solution by the help of a crimped glass rod. During free enzyme assay, solutions were directly added into tubes containing urease. A 5 min period was needed for the development of color reaction of 37°C. Then the solution was diluted to 10 mL and the absorbance was read at 625 nm by using a Spectro 22 Labomed spectrophotometer (Los Angeles, CA) against the blank.

Protein Determination

Protein measurements were carried out by the biuret method developed by Doumas et al. (*22*).

Water Uptake

Fibers of different graft yield (%) were allowed to stay in redistilled water for 48 h. The wet fibers were weighed after sandwiching between filter papers 10 times. Water uptake of the fibers were determined from the weights of dry (2 d/105°C) and wet samples.

Fiber Densities

A density-gradient tube containing carbon tetrachloride and xylene was used for density measurements. The densities were measured in relation to a glass float (Davenport, London, UK) of known density using a cathetometer (Precision Tool, Surrey, UK) with ± 0.01 mm sensitivity at 25°C.

Fiber Diameters

A Vanox-Olympus model microscope (Tokyo, Japan) was used to measure fiber diameters at 400–750× magnification. At least eight samples were used for each type of fiber.

Efficiency of Immobilization

Effect of pH on maximum activity was studied at pHs of 4–10 with acetate, phosphate, and carbonate buffers. Temperature variations vs maximum urease activity tests were performed at 25–75°C. Thermal inactivation was studied at 80°C. Operational stability (or reusability) was

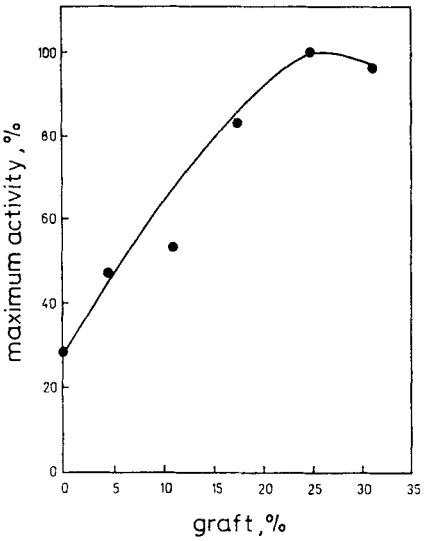


Fig. 1. Effect of acrylamide grafting on maximum urease activity of PET fibers. Urease concentration, 1 U/g fiber; GA concentration, 0.128*M*.

determined at 37°C. Storage stability of free and immobilized urease was detected at 4 and 25°C. Kinetic parameters were calculated from Lineweaver-Burk and Arrhenius equations. Absorbances obtained at the optimums for each experiment were taken as 100% maximum activity. Relative activity was calculated from the sample activity per immobilized enzyme unit and the optimum was taken as 100%.

RESULTS

Optimization of Immobilization

In order to investigate the effect of grafting on immobilization ratio of urease, ungrafted and 4.6 to 31.3% acrylamide-grafted PET fibers were used as support material. From Fig. 1, it is obvious that ungrafted fibers gave the lowest binding with urease molecules. After 4–5 assays corresponding to an h of operation at 37°C, no enzyme activity could be detected from the ungrafted fibers. The other observation found that, by increasing the graft yield to 25% Aam, the maximum activity drastically increased. Enzyme activity reached a saturation level at or around this graft value. Fibers of higher graft density (31.3%) did not show higher activity.

The optimum glutaraldehyde concentration for the activation of Aam-grafted PET fibers was found to be 0.128 mol dm^{-3} (Fig. 2). Glutaraldehyde concentrations higher than this value led to decrease in urease activity. Maximum enzymatic activity of glutaraldehyde-treated 25% Aam-g-PET fiber was about fivefold higher than untreated samples.

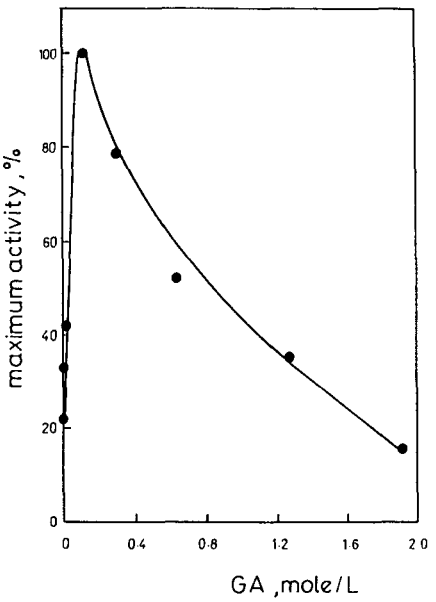


Fig. 2. Effect of glutaraldehyde concentration on maximum urease activity of 25% Aam-g-PET fibers. Urease concentration, 1 U/g fiber.

Figure 3 shows the maximum enzyme loading of 25% grafted fibers. Enzyme loading, that is, the amount of enzyme used during immobilization, gave an optimum at 1 U/g fiber, then the relative activity drastically decreased, although maximum activity still increased at higher enzyme concentrations.

Physical Properties of the Fibers

Water uptake, density, and diameter of ungrafted and grafted (4.6–31.3% Aam) fibers are given in Table 1. While the density of ungrafted fiber was 1.3749g/mL, grafted samples showed lower densities. Densities of 1.3741 and 1.3654 g/mL were measured for 4.6 and 31.3% grafted fibers, respectively. Table 1 also shows that the diameter and water uptake of fibers increased by increasing the graft yield. Ungrafted fibers had a mean diameter of 2.69×10^{-3} cm; the same value for 25% grafted fibers was 3.00×10^{-3} cm (Table 1 and Fig. 4). The samples with graft yield higher than 32% were not used during immobilization, since the fibers showed a rigid structure which could easily be broken up.

Properties of Immobilized Urease

The pH vs enzyme activity profiles of free and bound urease are given in Fig. 5. Both types of the enzyme gave optimum activity at pH 7.0. A broader range of pH stability was observed for the immobilized form.

Figure 6 shows the temperature optimums of free and bound urease. Free enzyme gave its maximum activity at 50–55°C, however, Aam-g-PET

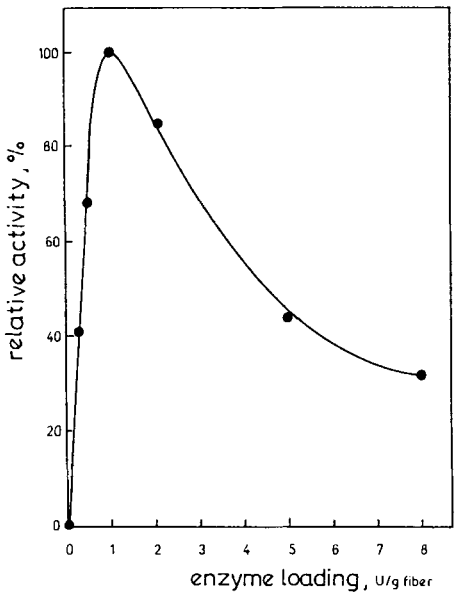


Fig. 3. Effect of urease loading on the relative activity of 25% Aam-g-PET fibers. GA concentration, 0.128M. Activity per immobilized enzyme unit obtained at optimum was taken as 100%.

Table 1
Physical Properties of PET Fibers

Grafting[a] time, min	Acrylamide graft yield, %	Water uptake (w/w), %	Density, g/mL	Diameter, cm × 10^{-3}
0	ungrafted	3.6	1.3749	2.6912
30	4.6	26.9	1.3741	2.7075
45	11.0	30.2	1.3736	2.7966
60	19.1	33.1	1.3732	2.9287
90	25.0	34.2	1.3689	3.0043
120	31.3	34.3	1.3654	3.1076

[a]Grafting conditions: Aam concentration, 40 g/L; Bz_2O_2 concentration, 9.68 g/L; and temperature, 75°C.

immobilized urease gave an optimum at 55–60°C, exhibiting higher stability to temperature.

Kinetic studies showed that the susceptibility of urease towards the substrate was slightly changed by immobilization. Table 2 gives the kinetic parameters derived from Lineweaver-Burk diagram and Arrhenius equations. K_m and V_{max} values of the bound enzyme were found as 4.50 mmol dm^{-3} and 98.0 mmol $s^{-1}g^{-1}$ protein, respectively. The K_m and V_{max} values for free urease were 2.82 mmol dm^{-3} and 32.3 mmol $s^{-1}g^{-1}$ protein, in the same order. The activation energy calculated for the immobilized urease was 14.14 kJ/mol, which was higher than the Ea of free urease (11.76 kJ/mol). The half-life values determined at 80°C also showed that urease immobilization protected the enzyme against heat inactivation.

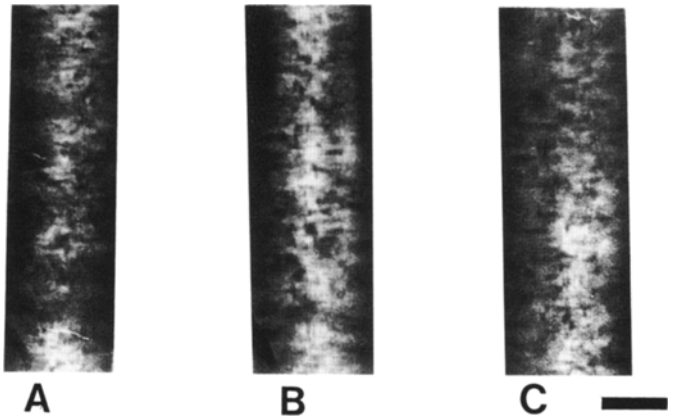


Fig. 4. Micrographs of ungrafted (**A**), and acrylamide-grafted [4.6% (**B**); 25.0% (**C**)] PET fibers (bar: 25 μm).

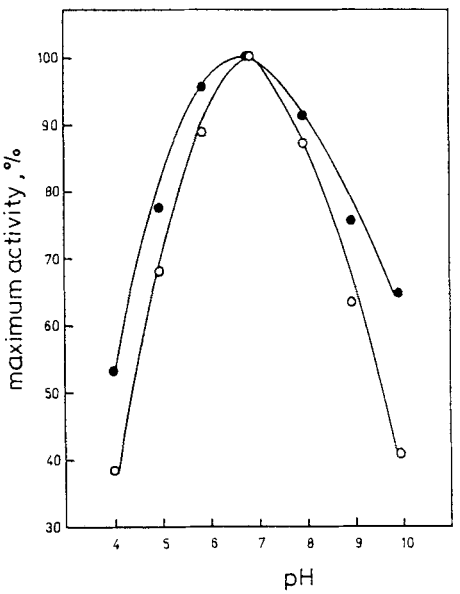


Fig. 5. Effect of pH on maximum activity of free (○) and bound (●) urease. Graft yield, 25% Aam; urease concentration, 1 U/g fiber; and GA concentration, 0.128*M*. Activity obtained at optimum pH was taken as 100%.

The operational stability and reusability of urease-bound graft fibers were found to be very high (Fig. 7). After 7 h of operation, which corresponds to 28 reuses in 90 d, the maximum activity of urease fibers settled at about 85%.

The storage stability of urease-containing fibers (25% grafted) at 4 and 25°C can be seen in Fig. 8. After 90 d, at 4°C, 92%, and at 25°C, 63% of the initial activities were determined for the fibers, while the free enzyme exhibited 46 and 21% of activity, respectively, after same period of time.

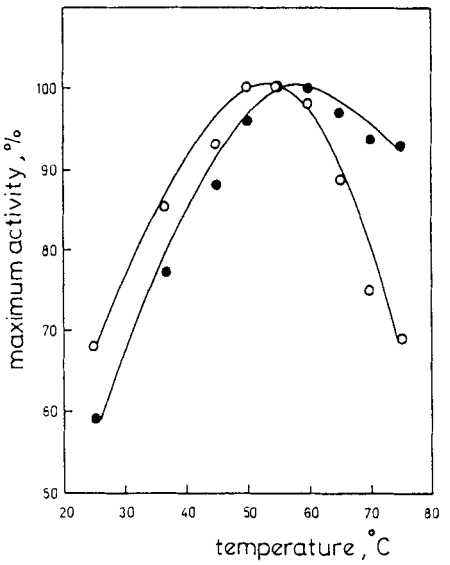


Fig. 6. Effect of temperature on maximum activity of free (○) and bound (●) urease. Graft yield, 25% Aam; urease concentration, 1 U/g fiber; and GA concentration, 0.128*M*. Activity obtained at optimum pH was taken as 100%.

Table 2
Kinetic Parameters of Free and Bound[a] Urease

Enzyme form	K_m, mmol/dm³	V_{max}, mmol/s g protein	E_a, kJ/mol	$t_{1/2}^{80°C}$[b], min
free	2.82	32.3	11.76	102
bound	4.50	98.0	14.14	225

[a]Immobilization conditions: 25% Aam-g-PET fiber; GA concentration, 0.128*M*; and enzyme concentration, 1 U/g fiber.
[b]Incubation time to reach 50% inactivation of enzyme at 80°C.

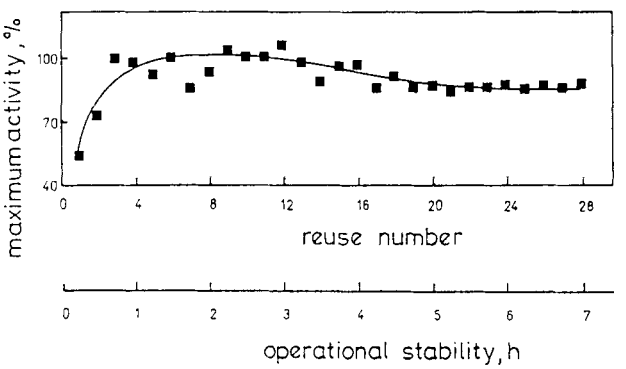


Fig. 7. Reusability and operational stability of 25% Aam-g-PET fibers. Urease concentration, 1 U/g fiber; GA concentration, 0.128*M*, incubation temperature, 37°C.

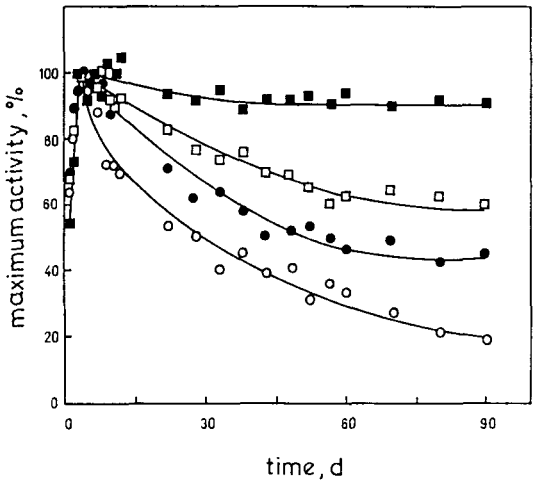
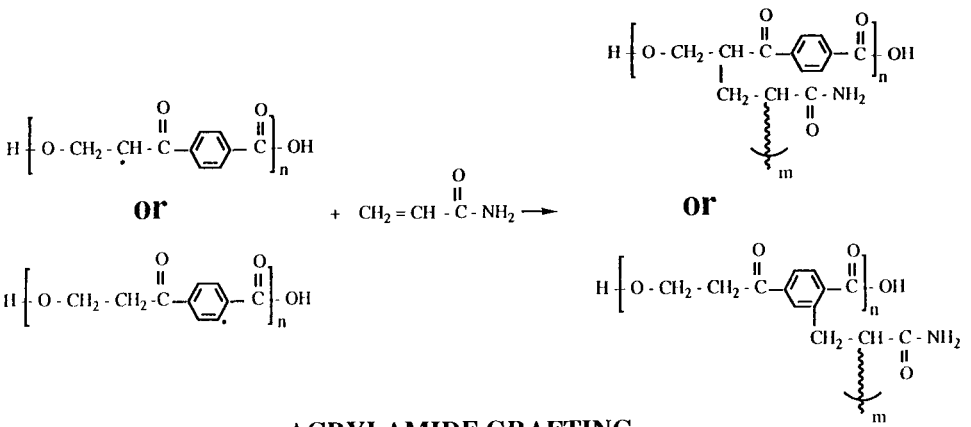


Fig. 8. Storage stability of free (●, 4°C; ○, 25°C) and immobilized (■, 4°C; □, 25°C) urease. Urease concentration, 1 U/g fiber; GA concentration, 0.128*M*.

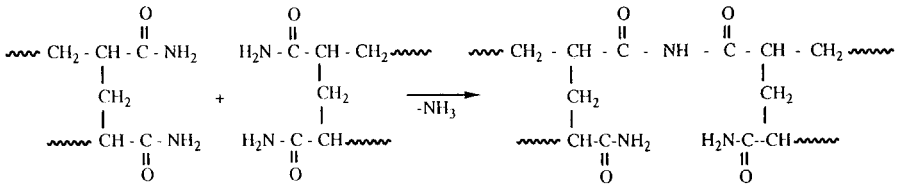


Structure 1

DISCUSSION

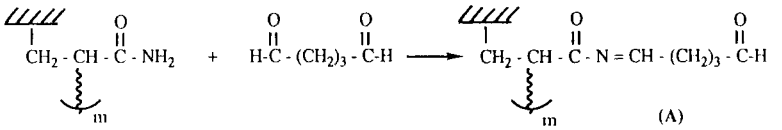
The high accessibility of functional groups fixed onto linear polymers bring many advantages. These grafted groups act as natural spacer arms, which are superior for enzyme immobilization purposes. Acrylamide grafting of PET fibers resulted in the addition of hydrophilic groups onto the chains (*see* Structure 1).

At high graft yields, the poly(acrylamide) chains were susceptible to crosslinking by releasing ammonia (23) as shown in Structure 2. The relatively low nitrogen content and the decrease in dye uptake with acidic dyes at high graftings (24) confirm this situation. The water uptake of the fibers reaches a constant level at 25–30% grafting, which also fits this event.

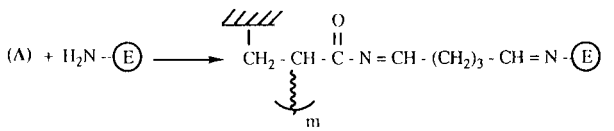


AMMONIA RELEASE FROM CROSSLINKED POLYACRYLAMIDE CHAINS

Structure 2



GLUTARALDEHYDE ACTIVATION



ENZYME COUPLING

Structure 3

Adsorption of the enzyme to the ungrafted or Aam-grafted PET fibers was very weak and, after a low number of assays, no urease activity could be observed showing that desorption occurred. In order to establish a stronger binding of the enzyme molecules (E) to the support, Aam-grafted fibers were activated by glutaraldehyde (*see* Structure 3).

Coupling of urease to glutaraldehyde may have been performed through the amine groups of the enzyme-forming amide bonds. The possibility of Schiff-base formation between amine and aldehyde groups should also be considered. The -SH group of urease may crosslink with glutaraldehyde after the amine groups have been used (*25*). It is important to notice that $-NH_2$ groups are more susceptible than -SH groups to reaction with glutaraldehyde.

Glutaraldehyde activation was effective in retaining high immobilization ratios, but high GA concentrations resulted in the inactivation of the enzyme molecules, probably by the binding of the active groups and/or intramolecular crosslinking of the molecules. The optimum GA concentration was found to be 0.128 mol/L; lower GA levels gave insufficient binding.

Hydrophilicity of the support strongly affects the enzyme activity (*9,26*). Since Aam grafting increased the hydrophilicity of PET fiber, the

increase in graft yield gave an increase in urease activity up to 25% grafting. It is obvious that this value gives the optimal steric conformation for support-urease binding and substrate-product diffusion. At higher graftings, because of the crosslinkages formed between glutaraldehyde and grafted chains, the number of functional groups that can bind to the enzyme molecules will decrease. Also, the enzyme molecules are faced with diffusional restrictions on their way to the functional groups.

The protein-binding capacity of 25% Aam-g-PET fibers was found to be 0.465 mg/g support, which corresponded to 1 U/g support. The assayed activity is the expressed activity of the bound urease, which usually is not the activity of the whole enzyme. Also, the amount of coupled enzyme is not directly proportional to the number of amine groups of the fiber, and some interactions between enzyme and free carboxyl groups of the support (due to hydrolysis of acrylamide) may have occurred. This type of binding gains irreversible character when the functional groups of the fiber and urease molecules have similar charges under certain pH conditions. This may be one of the reasons for the decrease in maximum activity during repeated assays.

The success of the immobilization of enzyme molecules can be determined by the structure of the grafted layer. Density, diameter, and water uptake of the fibers are such parameters for the characterization of the support structure. Our findings showed that the increase in graft yield caused a decline in fiber densities, as expected.

The increase in pH stability after immobilization depends on the nature of the binding and support used. Temperature optimum of the immobilized urease has been found to be 55–60°C, which is higher than the free enzyme optimum. Thermal stability experiments performed at 80°C also showed that urease–fiber preparations were much more stable than free urease. We can say that immobilization keeps the active site of the enzyme with less loss of inactivation against high temperatures. Similar results have been obtained in other studies (*27,28*). The stability of urease-fiber preparation can also be related to the multi-point attachment of the enzyme to the support.

During repeated assays, a twofold increase in maximum activity was observed between first and third assays. This is probably related to the cleavage of the weak bonds formed between the enzyme-active site and functional groups and/or to the conformational changes of the grafted side chains, leading to lower diffusion limitations for the substrate molecules. Similar results have been reported in other studies (*19,29*).

Apart from enzyme-binding capacity, variation of pH, and temperature characteristics, the operational and storage stability of the immobilized enzyme is of great importance, if the system is to be considered for biotechnological processes or biomedical purposes. After 7 h of continuous operation, the maximum activity settled at 85% of the initial activity at 37°C. Storage stability of the immobilized urease at 4 and 25°C was also significantly higher than that of the free enzyme. The small amount of

activity loss (15%) may be the result of inactivation of some of the enzyme molecules, or desorption of physically-adsorbed urease.

Kinetics parameters obtained after urease immobilization demonstrated that the enzymatic reaction nearly obeyed the Michaelis-Menten equations. The K_m, V_{max} and Ea values were slightly above the ones for free urease, showing that the steric and conformational modifications introduced by immobilization were not so wide and that the reduction of the substrate affinity towards the enzyme site was low. These findings were probably a result of the structure of the grafted fibers, which did not exhibit restricted diffusion of the substrate (*30*), and unfavorable conformation for the enzyme-substrate complex formation in the activated transition state (*31*).

CONCLUSIONS

The use of glutaraldehyde-activated Aam-g-poly(ethylene terephthalate) fibers as support material for urease immobilization has been demonstrated. It was found that the fibers were effective on urease thermostability and exhibited a very high operational stability. Graft yield and glutaraldehyde concentration were efficient on maximum enzyme activity. The results also indicated that the change in kinetic characteristics after immobilization was not so wide, suggesting that the binding did not cause an unfavorable conformation for product formation or diffusion restrictions. Studies on the design of grafted-fiber-based-urease and other enzyme reactors are in progress.

ACKNOWLEDGMENT

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