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Preparation and characterization of urease bound on crosslinked poly(vinyl alcohol)

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Abstract

Various parameters such as type and concentration of crosslinking agent, enzyme concentration, pH of the coupling medium and coupling time were optimized for the immobilization of urease on crosslinked poly(vinyl alcohol). Immobilized urease was thoroughly characterized for pH, temperature, solvents and storage stabilities and these properties were compared with the free enzyme properties. The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were calculated from Lineweaver–Burk plots for both free and immobilized enzyme systems. The energy of activation (ΔE_a) and the thermoinactivation constant (K_i) were calculated from the effect of temperature study. The urease-(PVA-F)-system shows good retention of activity even after 6–7 cycles. © 1998 Elsevier Science B.V.

Keywords: Urease; Immobilization; Preparation; Characterization; Stability

1. Introduction

In the recent studies of artificial kidneys, considerable attention has been focused on the development of a wearable or portable system [1,2]. Urea is one of the main toxic wastes in the dialyzate of hemodialysis. Since no efficient sorbent is available for urea [1,3], the most effective method for the removal of urea from an aqueous solution may be the utilization of urease [4]. At the current high cost of the enzyme urease, the most promising way of achieving economical feasibility is by using immobilized urease. Numerous synthetic and natural polymeric supports have been used for urease immobilization and their uses in medicinal and technical fields are well reported [5]. Some of the commonly used supports are carboxymethyl cellulose [6], polyurethane [7,8], poly(vinyl pyridine) [9], sepharose-2B [10], polyacrylamide [11], ion exchange resins [12] and copolymers of polyglycidylmethacrylate [13]. Styrene divinyl benzene [14], polypropylene [15] and poly(vinyl alcohol) [16], membranes have also been reported for urease immobilization. However, with many of these supports, immobilization has resulted in considerable reduction in enzyme activity as well as its binding capacity.

In the present paper we are reporting our studies on urease immobilization on crosslinked poly(vinyl alcohol). The major advantage of this polymeric support is that it can be prepared in

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any desirable polymorphic form such as film, bead, particle and powder with various porosities and surface areas. The urease immobilized on (-PVA-F-) was thoroughly characterized for pH, temperature, solvent, storage stabilities and kinetic studies.

2. Materials and methods

Crude crystalline urease (EC. 3.5.1.5) from jackbeans with 1 u/mg activity was obtained from TRIZMA Chemical (India) and urea from BDH (India). The poly(vinyl alcohol) crosslinked with paraformaldehyde was prepared in the bead form following the procedure reported by Chandy and Sharma [17]. All the other reagents used were of analytical grade and double distilled deionized water was used throughout the work.

2.1. Assay of enzyme activity

The urease solution was prepared in 50% glycerol. The activity of free as well as immobilized urease was determined by the phenol–hypochlorite method [18,19] using urea as a substrate in 0.2 M phosphate buffer of pH 8 and incubating the mixture at 37°C for 30 min. The resulting indophenol product was measured spectrophotometrically at 580 nm for the activity of urease. One unit of activity was defined as the amount of enzyme that liberates 1 μmol of ammonia under the conditions of assay.

2.2. Immobilization of urease

Different activation procedures were adopted for the immobilization of urease on PVA-F. Activation of crosslinked poly(vinyl alcohol) was done through hydroxyl groups using spacers *p*-benzoquinone [20], *p*-tolyl sulphonyl chloride [21] and cynuric chloride [22]. In the process of activation through cynuric chloride approx. 0.2 to 0.4 g of polymeric material was treated with 0.5 g of cynuric chloride in 10 ml dioxane at room temperature for 10 min.

Further it was treated with 25 ml of water and 25 ml of 20% acetic acid for 5 min. The resulting activated polymeric support was washed with water, acetone and 0.2 M phosphate buffer of pH 8 before immobilization of urease.

For the activation of -PVA-F- through *p*-benzoquinone ~ 0.2 to 0.4 g of support was stirred with 0.05 g *p*-benzoquinone dissolved in 10 ml of 20% ethanol, and 0.1 M phosphate buffer of pH 8 for 2 h at room temperature. The activated product was washed successively with 20% ethanol and water until the filtrate was free from benzoquinone and then with 1 M NaCl and 0.2 M phosphate buffer of pH 8 before immobilization.

During activation of polymeric support through *p*-tolylsulphonyl chloride (PTS), ~ 0.2 to 0.4 g -PVA-F- was activated for 1 h using 0.1 g PTS in 10 ml of dry dioxane at room temperature. To the reaction 1 ml of pyridine was added drop wise over 1 min. The polymeric support was washed with dry acetone, acetone:water (1:3) and 0.2 M phosphate buffer before immobilization.

The activated support was used for the immobilization of urease (2 ml of 7.5 mg ml^{-1}) in 0.2 M phosphate buffer of pH 8 at 278°K for 18 h. An INSREF low temperature shakerbath was used for the reaction to reduce the external diffusion barrier due to the formation of a Nernst layer. The immobilized product was washed with appropriate buffer and water. The Lowry assay procedure was adopted to determine the protein content of the supernatant liquid [23] and the activity of the coupled protein was measured by using the hypochlorite method [19] and urea as a substrate as described earlier.

The effect of the concentration of crosslinking agents, enzyme concentration, pH and coupling time on the extent of coupled protein and its retention of activity was studied through appropriate experiments.

2.3. Stability measurements

Comparison of pH, temperature, solvents and storage stabilities of immobilized urease with free urease was done through appropriate experiments. The kinetics of the enzymatic reactions was studied through Lineweaver–Burk plots.

3. Results and discussions

3.1. Activation of supports

Among the various enzyme immobilization techniques covalent coupling of the enzyme to the support is popular due to high accessibility and reusability of the bound enzyme [24]. In addition, no leaching of enzyme takes place during repeated uses as binding forces will be stronger than those in adsorption or ionic binding. Diffusion problems can be avoided during immobilization to a large extent. Protein loading can be controlled by varying activated groups at the polymer surface, etc.

In the present work we have examined covalent coupling of urease on the PVA-F support through different hydroxyl group activation methods. The results obtained are given in Table 1. It was observed that the urease-(PVA-F)-system shows moderate coupling with greater retention of enzyme activity through cynuric chloride activation. The immobilized system retained 70–80% urease activity of 8.2 u/g urease which was coupled to PVA-F at pH 8 in 0.2 M phosphate buffer. However, the other supports such as salicylic acid/resorcinol/formaldehyde resin and crosslinked chitosan did not show retention of enzyme activity after immobilization and hence were not used in further studies.

Various coupling conditions such as pH, coupling time and concentration of the enzyme were optimized for the better retention of urease activity and stability.

The effect of the pH of the coupling medium on the extent of immobilization of urease was studied in the pH range 5–10 using 0.2 M phosphate buffer. From the results the maximum coupling and retention of enzyme activity was observed to be at pH 8–9. The effect of time on the extent of immobilization was also studied by using various time intervals and the results obtained from the study show that almost quantitative coupling of urease takes place within 3–4 h reaction time with the 70–80% retention of activity. In addition, for the preparation of immobilized urease, the enzyme loading capacity was also calculated by using 25–150 u enzyme concentration per g of support. From the results, it was observed that the enzyme loading capacity increases as the concentration of enzyme increases up to 75 u/g support. Further increase in the urease concentration does not increase the extent of coupled protein but decreases the retention of enzyme activity. This may be attributed to the substrate diffusion limitation as well as with the increasing concentration of the enzyme on support, some of the active sites are either utilized in the coupling process or become inaccessible due to coiling of the enzyme. Similar results were obtained by us for pepsin [25].

Table 1
Immobilization of urease on crosslinked poly(vinyl alcohol)

Method of activation	(PVA-F)		
	protein coupled (u/g)	active protein (u/g)	retention of activity (%)
p-Benzoquinone	13.9	2.90	20
Cynuric chloride	8.20	5.75	70
p-Tolyl sulphonyl chloride	8.33	2.50	30

Time 18 h at 278 K in phosphate buffer pH 8; amount of supports used: 200–400 mg, enzyme used 15 mg.

3.2. pH activity profile

A change in optimum pH generally occurs upon immobilization depending on nature of the support used. This type of change is very useful in understanding the structure–function relationship of enzyme protein. Therefore, it is very useful to compare the activity of the free and immobilized enzyme as a function of pH. Changes in the enzyme structure result into change in the pH activity of the enzyme due to the change taking place in the microenvironment. However, in the present study of pH dependence of activity of free and immobilized urease it was observed that the urease-(PVA-F)-system has the same optimum pH as the free one (pH 8), but the pH activity profile is considerably widened due to diffusional limitations. Immobilized urease displayed a greater stability at higher pH values.

3.3. Temperature activity profile and thermal stability

The effect of temperature on the activity of free and immobilized urease was studied over 30–70°C. From the results, it was observed that at lower temperatures there is no significant difference in the activity of free and coupled enzyme. However, free enzyme loses its activity very rapidly above 50°C whereas the urease-(PVA-F)-system was thermally stable up to 70°C. The improved stability of the immobilized urease can be attributed to the covalent binding of the enzyme to the matrix.

The temperature dependence of chemical reactions is most frequently modelled in terms of the Arrhenius equation. The activation energy (ΔE_a) of free and immobilized urease was calculated from temperature–velocity data and is tabulated in Table 2. The observed higher activation energy for the immobilized urease than that for the free urease is due to the diffusion controlled reaction.

To study the thermal deactivation of the free and immobilized enzyme they were further heated at 60 and 70°C for a longer time duration. The plot A/A_o vs. time (where 'A_o', is the initial activity of the enzyme and 'A' is the activity after 't' min of temperature effect (Fig. 1) indicates that free enzyme loses its activity completely after 150 min incubation, whereas immobilized enzyme retained its activity over 3 h incubation time. From the results, the thermoinactivation constant (K_i, min^{-1}) was calculated by using the method proposed by Hayashi and Ikada [26] and the results are given in Table 2. An overall decrease in thermoinactivation was observed for the immobilized system even at 60 to 70°C indicating the improved thermal stability of the immobilized system.

3.4. Determination of Michaelis constant (K_m) and maximum reaction velocity (V_{max})

The rate of an enzyme-catalyzed reaction depends on the concentration of enzyme and substrate. The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) describes the reaction of an enzyme and substrate at lower con-

Table 2
Kinetic and thermodynamic parameters for free and immobilized urease

Sample	Michaelis constant (K_m) (mM)	Maximum reaction velocity (V_{max}) (mM min^{-1})	Thermoinactivation constant[a] (K_i) (min^{-1})		Activation energy[b] (ΔE_a) (kcal mol^{-1})
			60°C	70°C	
Urease-PVA-F	7.1×10^{-4}	2.43×10^{-5}	2.11×10^{-3}	3.40×10^{-3}	4.95
Urease	2.7×10^{-5}	3.4×10^{-5}	1.98×10^{-3}	2.04×10^{-3}	3.70

[a] $\ln A = \ln A_0 - K_i t$.
[b] $k = A \cdot e^{-\Delta E_a / RT}$.

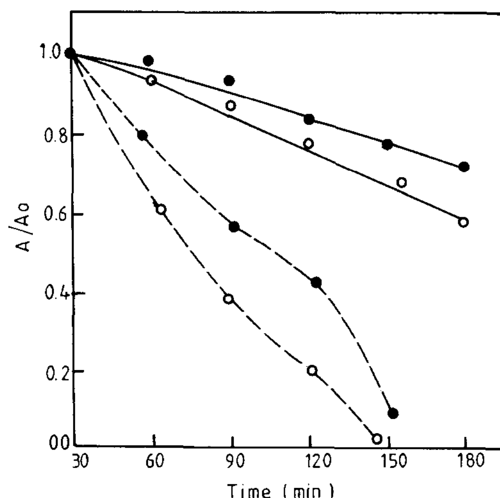

Fig. 1. Thermal stability for free and immobilized urease. 60°C: (●——●) – urease-PVA-F; (●- - -●) – urease; 70°C: (○——○) – urease-PVA-F; (○- - -○) – urease.

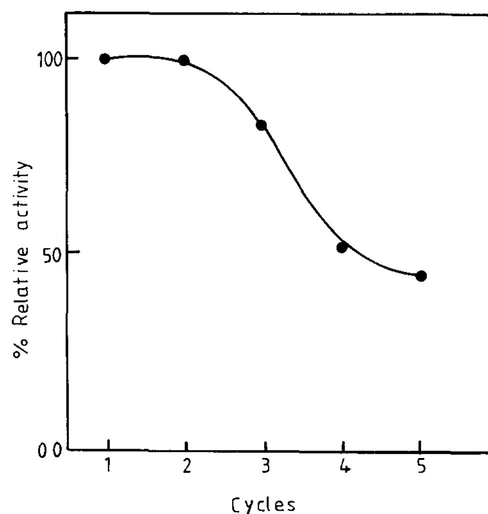

Fig. 2. Stability for repeated use. Temperature 37°C, pH 8 and time 30 min.

centrations and are expected to exhibit first order reaction kinetics. From Lineweaver–Burk plots, the Michaelis constant (K_m) and maximum reaction velocity (V_{max}) for free and immobilized urease were evaluated and are given in Table 2. It was observed that the higher apparent K_m and V_{max} values for urease-(PVA-F)-system indicate the limitation of diffusion resistance or partially kinetically controlled reaction. Such type of varied results were reported in earlier studies which originate due to the difference in techniques used, nature of support and source of the enzyme.

3.5. Stability studies

Stability of immobilized urease is very important in the continuous hydrolysis reactions. The results from Fig. 2 show that 50% residual activity of urease was retained even after 5 repeated cycles.

Storage stability of free and immobilized urease was determined by storing them at room temperature (30°C) in 0.2 M phosphate buffer of pH 8. From the results given in Fig. 3, it was observed that after 45 days free enzyme loses its activity completely whereas immobilized urease still retains 50% of its activity.

The activity and stability of immobilized enzyme in organic solvents is of significant importance in enzyme catalyzed organic synthesis. However, the reported stabilities of bound enzymes in organic solvents are highly variable and have been found to be dependent upon the

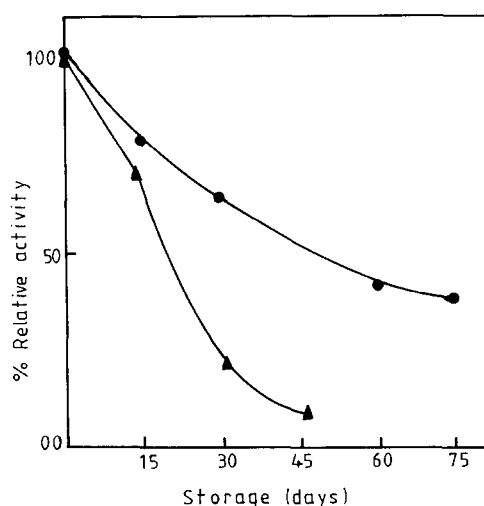

Fig. 3. Storage stability for free and immobilized urease. Temperature 37°C, pH 8. (●) urease-PVA-F; (▲) urease.

nature of enzyme itself as well as the nature of support and solvents [27]. Hydrolysis of urea by these enzyme systems was carried out in 1–5 M methanol, ethanol and propanol. It was observed that free and immobilized urease have similar stabilities towards water miscible solvents.

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