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Oriental Pharmacy and Experimental Medicine 2005 **5(1)**, 43-47

Immobilization of jack bean (*Canavalia ensiformis*) urease on gelatin and its characterization

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SUMMARY

Jack bean urease was immobilized on gelatin beads with the help of glutaraldehyde. The optimum immobilization (67.6%) was obtained at 30mg/ml gelatin concentration, 0.5 mg/bead enzyme protein concentration, 1% glutaraldehyde and at 4°C incubation temperature. The $t_{½}$ of immobilized urease was approximately 90 days at 4°C compared with t½ of 20 days for the soluble urease, under identical condition. The apparent optimum pH shifted from 7.3 to 8.0 when the urease was immobilized. The optimum stability temperature of immobilized urease was found to be 60°C while that of soluble urease was 45°C. Time-dependent thermal inactivation studies showed monophasic kinetics for soluble urease and immobilized urease at 70°C, respectively. The immobilized urease beads stored at 4°C showed practically no leaching over a period of 30 days. Here we are presenting an easy and economical way of immobilizing urease on the gelatin beads making it suitable for various applications.

Key words: Urease; Immobilization; Jack bean; Gelatin; Urea estimation

INTRODUCTION

Recovery of enzymes from reaction solutions and separation of the enzymes from substrates and products are in general very difficult. These problems can be successfully tackled by immobilization of the enzyme. Gelatin has been used as a convenient material for the immobilization of proteins and enzymes. Gelatin, a product of hydrolysis of collagen hardens with some aldehydes and chromium salts. It has reactive groups primarily composed of hydroxyl, carboxyl and amino functions, were used as support material for immobilization of several enzymes and viable cells of *Saccharomyces* sp. (Elcin and Akbulut, 1992; Dinckaya and Telefoncu, 1993). The cost of material with respect to others used as the supports is low. Formaldehyde and glutaraldehyde cross-linkers are expected to react with amino groups of gelatin to immobilize urease by forming a cross-link between gelatin and glutaraldehyde.

Immobilization of urease has been carried out in diverse matrices and immobilized urease has analytical and clinical applications (Das *et al.,* 1997; Das *et al.,* 1998; Das and Kayastha, 1998; Kayastha and Srivastava, 2001; Kayastha *et al.,* 2003; Reddy *et al.,* 2004) and possible future potential for the treatment of urea-containing effluents (Kamath *et al.,* 1988; Kamath and D'Souza, 1991). Urease from *Arthrobacter mobilis* has been used for removal of urea from fermented beverages, such as sake (Miyagawa *et al.,* 1999).

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MATERIALS AND METHODS

Material

Gelatin, glutaraldehyde, dialysis tubing, BSA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade. All buffers and reagents were prepared in Millipore water. Jack bean urease (EC 3.5.1.5) was purchased from Sigma Chemicals, USA.

Soluble urease assay

Assay was done by determining the amount of ammonia liberated in a fixed time interval on incubating the enzyme and urea as described earlier (Das *et al*., 2002). The amount of ammonia liberated in a test solution was calculated by calibrating Nessler's reagent with standard NH_4Cl solution. Absorbance was measured at 405 nm on a Unicam UV-Vis Spectrophotometer. An enzyme unit has been defined as the amount of enzyme required to liberate 1 μmol of ammonia per min under our test conditions (0.1 M urea, 0.05 M Trisacetate buffer, pH 7.3, 37°C).

Protein assay

Protein content of soluble urease and in the washings of immobilized urease was assayed by the method of Lowry *et al.* (1951), using BSA as standard.

Preparation of gelatin gel

1.5 g of gelatin was dissolved in 50 ml Tris- buffer (50 mM, pH 7.3) by heating at 60°C with continuous stirring for 1 hr to get clear solution. This solution was allowed to cool and the solidified mixture was stored at 4°C. Prior to immobilization, mixture was heated up to 60°C and then brought to room temperature to obtain a clear solution.

Urease immobilization

Clear solution of gelatin (30 mg/ml), 0.5 mg/ml enzyme and 1% glutaraldehyde were added, respectively and stirred for 10 sec. Immediately, the mixture was cast in hollow plastic cylinders (0.5 cm dia. x 0.5 cm ht.) and kept at 4°C for 24 hr. Beads were then washed with 50 mM Tris-acetate buffer, pH 7.3 to remove any unbound enzyme. Beads were stored at 4°C in 50 mM Tris-buffer, pH 7.3.

Assay of immobilized urease

The beads were incubated in 1 ml assay buffer (0.05 M Tris-acetate buffer, pH 8.0), at 37°C with 1 ml urea (0.1 M) with intermittent shaking. After the desired period of incubation, an aliquot of 1.0 ml, from the reaction mixture was withdrawn and assayed as described earlier (Das *et al*., 2002). Percentage immobilization was defined as the (total activity in immobilized beads/total activity of the soluble enzyme loaded) x 100.

Steady state kinetics

The optimum pH for the immobilized urease was determined by varying the pH of assay buffers. The enzymatic activity was determined for each buffer by the method described above. Optimal temperature was studied by varying the temperature of immobilized urease; the activity of immobilized urease was assayed at increasing temperature ranging from 20°C to 80°C.

Storage stability of immobilized urease

For storage stability studies, immobilized beads were kept at 4°C. The activity of immobilized urease was determined on different days by the method described above. For each assay fresh beads were taken.

Thermal inactivation of immobilized urease

Around 25-30 beads were incubated in assay buffer (0.05 M Tris-acetate, pH 8.0) at desired temperature (70°C). Two beads were withdrawn at specified time intervals, allowed to cool and transferred immediately to the assay solution (total volume 2 ml; containing 1 ml, 0.05 M Tris-acetate buffer, pH 8.0 and 1 ml, 0.1 M urea). Residual activity was determined by the usual assay method at 37°C.

RESULTS AND DISCUSSION

Urease immobilization

The general behaviour of urease immobilized on gelatin under different conditions of immobilization was studied. Different concentrations of gelatin and loading volume of enzyme were used in order to gain maximum % immobilization with minimum leaching of the enzyme. Table 1 shows details of the different conditions used. It is clear from the data of table that the optimum immobilization (67.6%) was obtained at 0.5 mg of protein per bead at 4℃. The gelatin and glutaraldehyde concentrations were 30 mg/ml and 1%, respectively. The percent immobilization reduced with decrease in protein concentration below 0.5 mg/bead and with increase in temperature from 4°C to 27°C. An increasing concentration of glutaraldehyde above 1% leads to a rapid polymerization of gelatin-enzyme mixture and hence creates practical problem in gel casting. On decreasing the concentration of glutaraldehyde below 1%, the beads were too soft and fragile and thus difficult to handle for repeated use.

Storage stability of beads

The stability of urease enhanced quite significantly upon immobilization. The $t_{½}$ of immobilized urease approximately 90 days when was kept in 0.05 M Tris-acetate buffer, pH 7.3 at 4°C compared with $t_{½}$ of 20 days for the soluble urease under identical condition. The beads showed linearity with respect to the activity indicating the homogenous distribution of the enzyme in the polymer. Storage stability ($t_{½}$) of immobilized pigeonpea urease on flannel cloth, alginate, chitosan and DEAE-cellulose paper strip were 70, 75, 110 and 150, respectively at 4°C (Das and Kayastha, 1998; Das *et al*., 1998; Kayastha and Srivastava, 2001; Reddy *et al*., 2004). The immobilized urease showed practically no leaching of enzyme (less than 2%) over a period of 30 days. Enhanced thermal stabilities have been reported for covalently bound jack bean urease on nylon (Sundaram and Hornby, 1970).

Steady state kinetics

The effect of pH on the activity of free and immobilized urease is shown in Fig. 1. The pH optima of the soluble and immobilized urease were 7.3 and 8.0, respectively in 50 mM Tris-acetate buffer. There was a shift of 0.7 units toward the basic side resulting from the binding of the enzyme. Jack bean urease immobilized on porous glass beads and molecular sieve 4A showed a shift toward the acidic side (Weetal and Hersh, 1969; Iyenger *et al*., 1982) however, for jack bean urease immobilized on a fixed bed reactor showed a shift towards basic side (Moynihan *et al*., 1989). Result of the effect of temperature on gelatin bound urease is shown in Fig. 2. Soluble urease from jack bean has an optimum temperature of 45°C, whereas gelatin bound urease was stable up to 60°C. There is a significant increase in optimum incubation temperature when urease is bound to gelatin, thus indicating that the immobilized urease resist

Table 1. Optimum conditions for the immobilization of urease on gelatin at 4°C

Gelatin (mg/ml)	Enzyme (mg/bead)	Glutaraldehyde (%)	Immobilization (%)
20	0.5	1.0	60.2
30	0.5	1.0	67.6
40	0.5	1.0	52.3
30	0.4	1.0	50.8
30	0.3	1.0	47.2
30	0.5	0.5	36.9
30	0.5	1.5	42.5
30	0.5	2.0	27.8

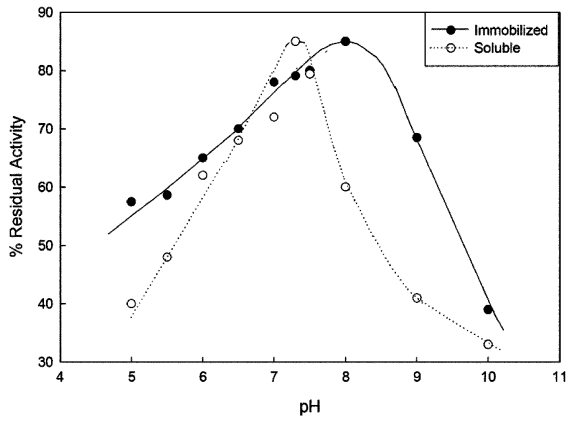

Fig. 1. Effect of pH on soluble and gelatin-immobilized urease.

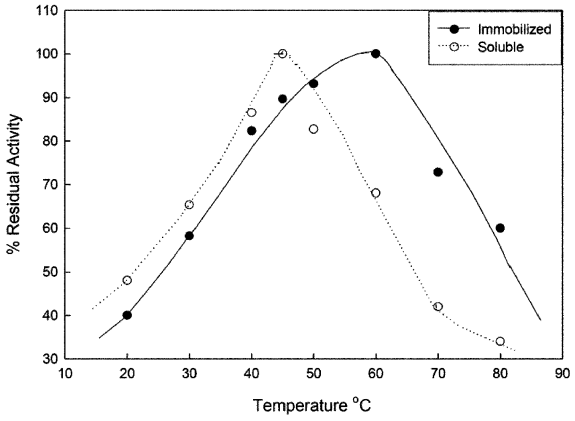

Fig. 2. Effect of temperature on the soluble and gelatin-immobilized urease.

denaturation. A similar shift in optimum temperature was observed with chitin bound lactase and jack bean urease on sieve 4A, where even up to 65°C activity increased (Stanley *et al.*, 1976; Iyenger *et al.*, 1982). Time-dependent thermal inactivation studied with soluble urease and immobilized urease at 70°C; both showed monophasic kinetics i.e., complete enzyme activity was lost in a single phase (Fig. 3). Recently, DEAE-cellulose-immobilized urease from pigeonpea also showed similar type of kinetics (Reddy *et al.*, 2004).

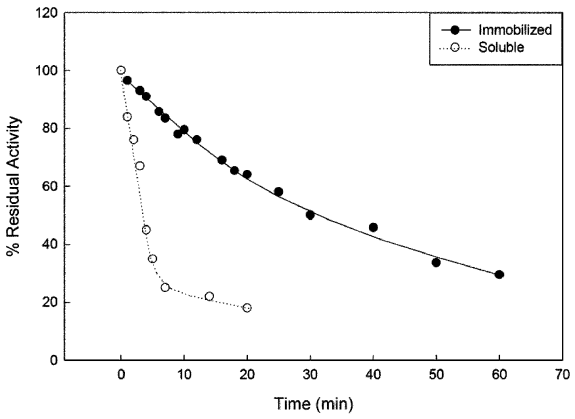

Fig. 3. Effect of thermal inactivation on soluble and gelatin-immobilized urease at 70°C.

ACKNOWLEDGEMENTS

Financial support from Council of Scientific and Industrial Research, New Delhi (Junior Research Fellowship to S.K.) is gratefully acknowledged. We thank Professor O.P. Malhotra for critical reading of this manuscript.

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