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Pigeonpea (*Cajanus cajan* L.) Urease Immobilized on Glutaraldehyde-Activated Chitosan Beads and Its Analytical Applications

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Abstract

Urease from pigeonpea (*Cajanus cajan* L.) was covalently linked to crab shell chitosan beads using glutaraldehyde. The optimum immobilization (64% activity) was observed at 4°C, with a protein concentration of 0.24 mg/bead and 3% glutaraldehyde. The immobilized enzyme stored in 0.05 *M* Tris-acetate buffer, pH 7.3, at 4°C had a $t_{1/2}$ of 110 d. There was practically no leaching of enzyme (<3%) from the immobilized beads in 30 d. The immobilized urease was used 10 times at an interval of 24 h between each use with 80% residual activity at the end of the period. The chitosan-immobilized urease showed a significantly higher Michaelis constant (8.3 m*M*) compared to that of the soluble urease (3.0 m*M*). Its apparent optimum pH also shifted from 7.3 to 8.5. Immobilized urease showed an optimal temperature of 77°C, compared with 47°C for the soluble urease. Time-dependent kinetics of the thermal denaturation of immobilized urease was studied and found to be monophasic in nature compared to biphasic in nature for soluble enzyme. This immobilized urease was used to analyze blood urea of some of the clinical samples from the clinical pathology laboratories. The results compared favorably with those obtained by the various chemical/biochemical methods employed in the clinical pathology laboratories. A column packed with immobilized urease beads was also prepared in a syringe for the regular and continuous monitoring of serum urea concentrations.

Index Entries: Urease; pigeonpea; *Cajanus cajan*; chitosan; immobilization; urea estimation.

Introduction

The entrapment of a biocatalyst within gel structures such as calcium alginates *(1,2)* is one of the least disruptive methods of immobilization,

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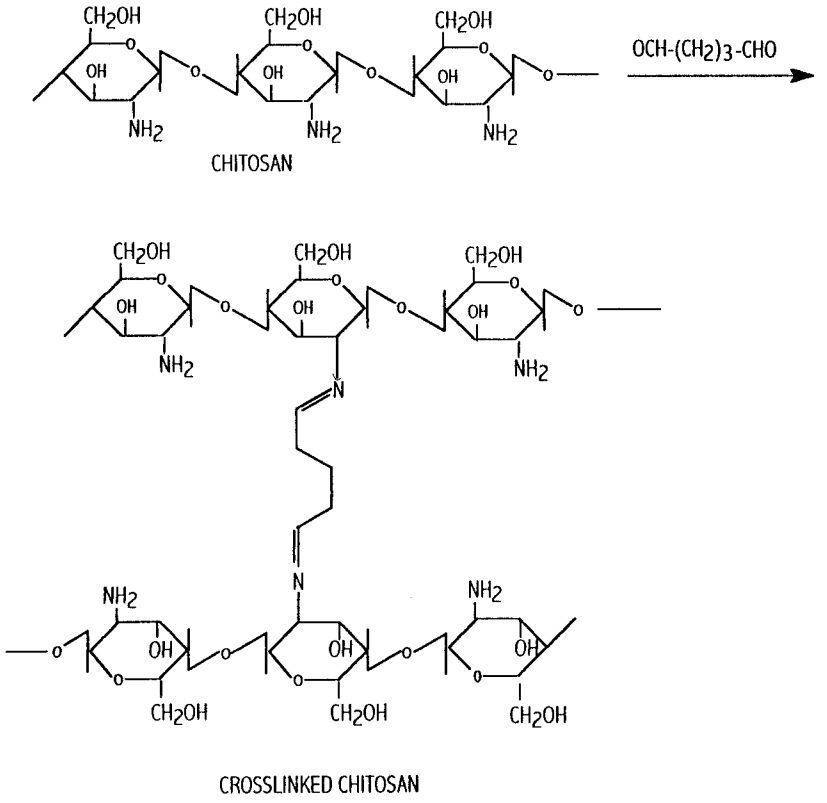

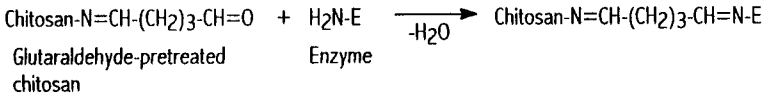

Fig. 1. Cross-linking of chitosan with glutaraldehyde and its subsequent attachment to the enzyme moiety.

yielding resilient beads with high retention of activity. However, low diffusional constants of substrates in the gel have been reported *(3)*. In addition, biocatalyst leakage may lower the performance and complicate control of the process. Covalent linkage of enzyme to matrix overcomes these problems and offers the advantage of increased stability and an increase in the degree of percentage of immobilization, provided that the functional group involved in linkage is not of active site residues.

Chitosan is a polysaccharide mainly made up of 2-amino-2-deoxy-D-glucose units, which are joined by β-1,4-linkages. It is obtained by deacetylation with drastic alkaline treatment of chitin, which is the principle component in the exoskeletons of crustaceans and insects. The cost of this material with respect to others used as a support is low and, thus, is

used for immobilization of some enzymes *(4,5)*. Enzyme immobilization on chitosan can mainly be achieved by means of the glutaraldehyde reaction between the free amino groups of chitosan and the enzyme molecules to form a covalent linkage *(4)* (Fig. 1). This biopolymer, stable under pH 5.5, can form gels by acting as a polycation with sodium triphosphate as an oppositely charged electrolyte *(6)*, which permits one to obtain matrices in different shapes and sizes.

Recent investigations have focussed on the use of immobilized urease in kidney machines for blood detoxification *(7,8)*, because approximately half a million patients worldwide are being supported by hemodialysis *(9)*. There is at present much research in in vivo sensing of urea by developing implantable biosensors and reducing the size of dialysis machines (portable) by using immobilized urease, in order to help patients know when they need dialysis, as well as to monitor the process themselves *(10)*.

Several biosensors with immobilized urease have been described for blood urea assay in cases of kidney malfunctioning. These include a recent method of entrapment of urease inside reverse micelles as a method of immobilization and using a glass electrode as a biosensor *(11–13)*. Immobilization of urease has been carried out in diverse matrices *(1,14–17)*, and immobilized urease has analytical and biomedical applications and possible future potential for the treatment of urea-containing effluents *(1,15,18,19)*.

In the present study, we attempted to immobilize urease isolated from pigeonpea (*Cajanus cajan* L.) on chitosan beads and compared the kinetic properties of soluble urease with those of immobilized enzyme. The immobilized urease was employed for assay of blood urea in clinical samples. Furthermore, a packed-bed reactor of immobilized beads was also designed for continuous monitoring of urea in biological samples, in this study.

Materials and Methods

Enzyme

Urease was isolated from dehusked seeds of pigeonpea (*C. cajan* L.) procured from the local market and purified as described earlier by Kayastha et al. *(20)*. Purified enzyme was about 95% pure as judged by native polyacrylamide gel electrophoresis (PAGE)/sodium dodecyl sulfate-PAGE.

Chemicals

Crab shell chitosan and glutaraldehyde were obtained from Sigma (St. Louis, MO). Tris buffer, phosphate buffer, urea, and trichloroacetic acid were from SRL (India). Nessler's reagent was from Hi-Media (India). Serum samples were collected from a local pathology laboratory. All reagents were of analytical grade. All the reagents were prepared in double-distilled water from an all-glass (Corning) assembly.

Preparation of Chitosan Beads

Chitosan (1.5 g) was dissolved in 50 mL of aqueous solution of 2.5% (v/v) acetic acid by heating at 60°C with continuous stirring for 2 h. This solution was added to a gently stirred 1.5% (w/v) 100-mL sodium triphosphate solution using a syringe (3 mL) to obtain beads of uniform shape and size. Before activation, beads were washed to neutrality and suspended in 0.025 *M* KH_2PO_4 and 0.025 *M* Na_2PO_4 buffer, pH 6.86, as described previously *(21)*.

Immobilization of Urease

The chitosan beads (support) were activated by treating them with 1, 2, 3, and 5% concentrations of glutaraldehyde in 0.025 *M* phosphate buffer, pH 6.86, and allowed to stand for 24 h at room temperature (27°C). After activation, the beads were washed thoroughly with the above buffer to free it from glutaraldehyde. Beads were then treated with the desired protein concentration of enzyme, and the preparation was left overnight at 4°C and room temperature. The next day, the beads were washed with the same buffer to remove any unbound enzyme. Beads were stored in 0.05 *M* Tris-acetate buffer, pH 7.3, at 4°C. The percentage of immobilization was calculated as follows: (Total activity obtained in beads after immobilization/ Total activity of the soluble enzyme loaded) × 100.

Construction of Column of Chitosan Beads for Continuous Monitoring of Urea

Approximately 10–12 beads were allowed to pack under gravity in a 2-mL syringe (height: 2 cm; diameter: 1 cm). The bottom of the syringe was connected to a one-way stopcock to which a thin tubing was connected. A plastic grid was put on the top so that the bed top was not disturbed and another at the bottom so that any bead or part of a bead could not escape from the column. The column was washed with several bed volumes of 0.05 *M* Tris-acetate buffer, pH 7.3, and then stored at 4°C with some buffer.

For continuous monitoring of urea in biologic samples, buffer was removed from the column. Deproteinized serum (0.2 mL) and buffer (0.8 mL) were added to the column and allowed to stand for 20 min at room temperature. The total reaction mixture was collected in a 50-mL measuring flask after 20 min by opening the stopcock. Color was developed as described in the next section for soluble enzyme.

Enzyme Activity Assay

For routine measurement of soluble urease activity, ammonia liberated at a fixed time interval at an enzyme saturating concentration of urea was determined using Nessler's reagent, as described earlier *(1)*. The yellow color produced was measured spectrophotometrically at 405 nm. The amount of ammonia liberated in the test solution was calculated by cali-

brating the reagent with standard ammonium chloride solutions. One enzyme unit was defined as the amount of enzyme required to liberate 1 μmol of ammonia/min under our test conditions (0.05 *M* Tris-acetate buffer, pH 7.3, containing 0.1 *M* urea, at 37°C).

For assay of immobilized enzyme, the beads were incubated in 0.05 *M* Tris-acetate buffer, pH 8.5, at 37°C with 0.2 *M* urea with intermittent shaking. After the desired incubation time, a 1.0-mL aliquot of the reaction mixture was withdrawn for color development as just described for soluble urease.

Assay of Blood Urea with Immobilized Urease

Three to four beads were preincubated in 0.8 mL of assay buffer (50 m*M* Tris-acetate, pH 8.5) for 5 min at 37°C. The reaction was started with 0.2 mL of serum. A 0.5-mL aliquot of the reaction mixture was separated after the 20 min of incubation. This 0.5-mL reaction mixture was treated with 0.5 mL of 10% trichloroacetic acid; precipitated proteins were removed by centrifugation. Color was developed from the resultant supernatant as described in the previous section.

Protein Assay

Protein was assayed by the method of Lowry et al. *(22)* by using Folin-Ciocalteu's reagent calibrated with standard bovine serum albumin protein. The amount of protein immobilized was estimated by subtracting the amount of protein determined in the supernatant after immobilization from the total amount of protein used for the immobilization.

Storage Stability of Immobilized Preparation

For storage stability studies, immobilized urease beads were kept at 4°C. The activity of immobilized urease was determined on different days by the method described earlier. After each assay, enzyme beads were washed with buffer and stored at 4°C for further use.

Determination of Optimum pH

The optimum pH for the immobilized urease was determined by increasing the pH of the assay buffer from 5.5 to 9.0. The enzymatic activity was determined for each buffer by the method described in above sections. For each pH tested, fresh beads were used. All further studies were carried out at optimum pH.

Determination of Michaelis Constant (K_m)

The immobilized urease was assayed at increasing concentrations of substrate urea from 1 to 100 m*M* as described earlier. Fresh beads were used for each substrate concentration. K_m was determined using the Lineweaver-Burk method.

Determination of Optimum Temperature

The activity of immobilized urease was assayed at increasing temperature ranging from 10 to 90°C. Fresh chitosan beads were used for each assay at different temperatures.

Thermal Inactivation of Immobilized Urease

Twenty-five to 30 beads were incubated in assay buffer (0.05 *M* Tris-acetate, pH 8.5) at the desired temperature (95°C). Two to three beads were withdrawn at specified time intervals, cooled, and transferred immediately to the assay solution (2.0 mL, containing 1.0 mL of 0.05 *M* Tris-acetate buffer, pH 8.5, and 1.0 mL of 0.2 *M* urea). Residual activity was determined at 37°C by the method described earlier.

Results and Discussion

Immobilization of Urease

The general behavior of urease immobilized on chitosan beads under different conditions of immobilization was studied. The percentage immobilization with varying glutaraldehyde concentration, protein concentration, and incubation temperature were determined (see Table 1).

It is clear from the results in Table 1 that the optimum immobilization (64%) was obtained at 0.24 mg/bead protein concentration, 3% glutaraldehyde, and 4°C incubation temperature. The enzymatic activity of immobilized urease decreased with a decrease in protein concentration below 0.24 mg/bead and with an increase in temperature above 4°C. An increase or decrease in glutaraldehyde concentration from 3% also led to decreased activity. Furthermore, with an increase in glutaraldehyde concentration, fragility of beads was observed. Iyengar et al. *(23)* have also used 2.5–3% as the optimal concentration of glutaraldeyde for immobilization of jack bean urease on molecular sieve 4A.

Storage Stability of Beads

The immobilized enzyme was quite stable, with a $t_{1/2}$ of approx 110 d when kept in 0.05 *M* Tris-acetate buffer (pH 7.3) at 4°C, compared with a $t_{1/2}$ of 31d for the soluble urease under identical conditions, as shown in Fig. 2. Furthermore, the storage stability of chitosan beads was much better compared to urease immobilization shown earlier by us, 70 and 75 d for cotton and alginate beads, respectively *(1,15)*. The beads showed good linearity with respect to activity, indicating the homogeneous distribution of the enzyme to the polymer (data not shown). The immobilized urease showed practically no leaching of enzyme (<3%) over a period of 30 d.

When the same beads were repeatedly used 10 times at an interval of 24 h. between each use, for the blood urea estimation, they still showed 80% residual activity.

Table 1
Optimum Conditions for Immobilization of Urease on Chitosan Beads

Protein concentration (mg/bead)	Glutaraldehyde concentration (%)	Incubation temperature (°C)	Immobilization (%)
0.24	1	4	19
0.24	2	4	56
0.24	3	4	64
0.24	5	4	53
0.18	3	4	51
0.12	3	4	43
0.24	3	27	41

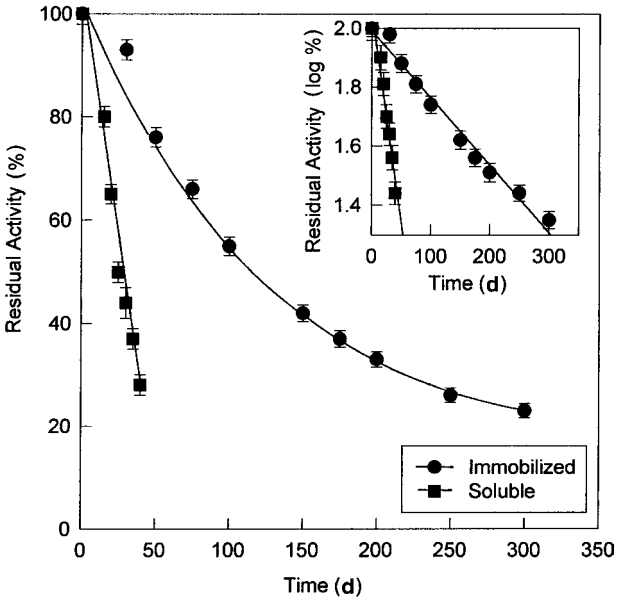

Fig. 2. Shelf-life of urease immobilized on chitosan vs soluble urease. Inset shows the semi-log plot for the same (see Materials and Methods for the assay of immobilized and soluble urease activity).

Effect of pH on Immobilized Urease

The behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution can have a different pH optimum from the same enzyme immobilized on a solid matrix. Depending on the surface and residual charges on the solid matrix and the nature of the enzyme bound, the pH value in the immediate vicinity of the enzyme molecule may change, thus causing a shift in the pH optimum of the enzyme activity *(24)*.

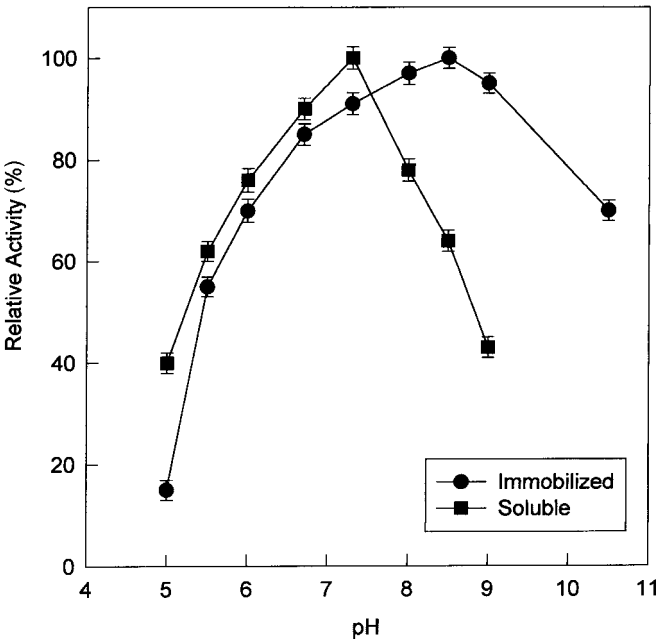

Fig. 3. Effect of pH on chitosan-immobilized urease vs soluble urease.

The effect of pH on the activity of free and immobilized urease is given in Fig. 3. The pH optima of the soluble and chitosan bound urease were 7.3 and 8.5, respectively. There was a shift of 1.2 U toward the basic side resulting from the binding of urease. A similar shift has been observed for jack bean urease immobilized on a fixed-bed reactor *(25)*. In this case, the shift was observed from 6.6 (soluble) to 7.6 (immobilized) in dialysate solution. However, jack bean urease immobilized on porous glass beads and molecular sieve 4A showed a shift toward the acidic side *(23,26)*.

Effect on Michaelis Constant (K_m)

Urease immobilized on chitosan beads showed an apparent K_m value of 8.3 m*M*, which is approximately three times higher than the soluble urease (3 m*M*; data not shown). It is postulated that an unstirred layer of solvent surrounds suspended water-insoluble particles. This unstirred layer is known as the "Nernst layer," and with water-insoluble enzymes (i.e., immobilized enzymes), a concentration gradient of substrate is established across the layer. Consequently, saturation of an enzyme attached to a water-insoluble particle will occur at a higher substrate concentration than normally required for the saturation of the freely soluble enzyme, thus leading to an increase in the K_m value.

A slightly higher apparent Michaelis constant was found for jack bean urease immobilized in polyacrylamide gel (5 m*M*) *(27)*. However, urease

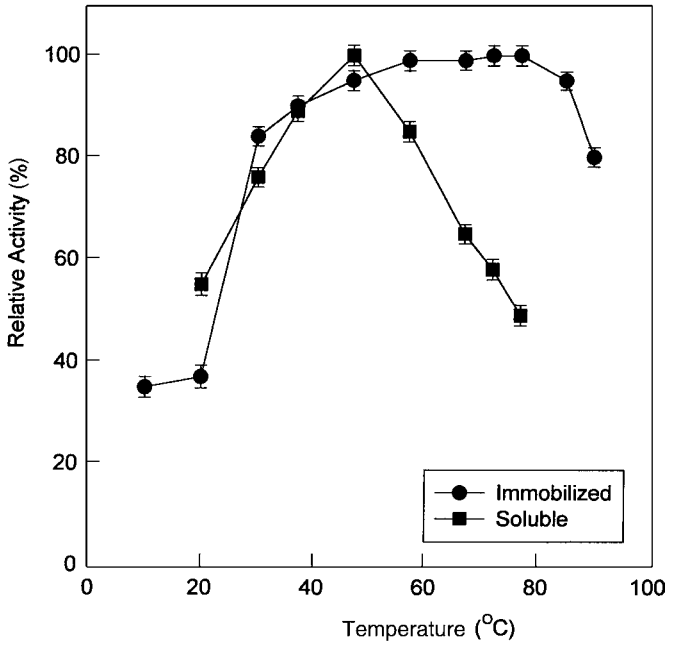

Fig. 4. Effect of incubation temperature on chitosan-immobilized urease vs soluble urease.

immobilized in a hydrocarbon-based liquid surfactant membrane exhibited approx 50 times greater K_m than the soluble enzyme *(28)*.

Effect of Temperature on Immobilized Urease

Results of the effect of temperature on chitosan-bound urease and soluble urease are shown in Fig. 4. Soluble urease from pigeonpea has an optimum temperature of 47°C, whereas chitosan-bound urease was stable up to 77°C. There is a significant increase in the optimum temperature when urease is bound to chitosan, thus indicating that the immobilized urease resists denaturation. Note that under normal conditions of activity in living cells, almost all enzyme exists in a membrane-bound form or is bound to other macromolecules and is rarely found in the free state as in in vitro experiments *(24)*. A similar shift in optimum temperature was observed with chitin-bound lactase and jack bean urease immobilized on sieve 4A, where even up to 65°C activity was found to increase *(23,29)*. Enhanced thermal stabilities have been reported for numerous covalently bounded enzymes (e.g., jack bean urease) on nylon *(30)*.

Time-Dependent Thermal Inactivation of Immobilized Urease

The time-dependent thermal inactivation kinetics for both the soluble and chitin-bound urease are shown in Fig. 5. The time-dependent thermal

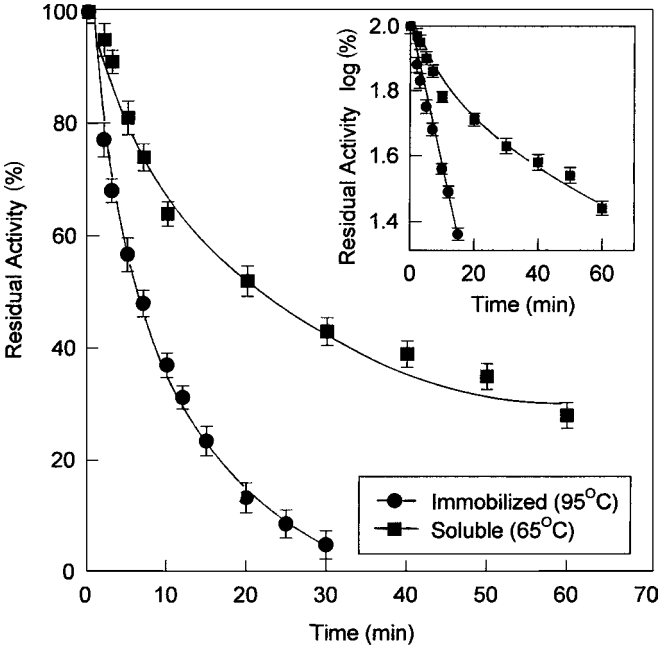

Fig. 5. Time-dependent thermal inactivation of chitosan-immobilized urease vs soluble urease. Inset shows the semi-log plot for the same.

inactivation of soluble pigeonpea urease at 65 and 70°C showed biphasic kinetics (data shown only at 65°C), in which half of the initial activity was destroyed more rapidly than the remaining half *(31)*, thus giving rise to biphasic kinetics, which could be described by a two-term equation:

$$A_t = A_{\text{fast}} \cdot e^{-k_{\text{fast}} \cdot t} + A_{\text{slow}} \cdot e^{-k_{\text{slow}} \cdot t}$$

where A_t is the percentage of residual activity at time t, A_{fast} and A_{slow} are the amplitudes (expressed as percentage of initial activity), and k_{fast} and k_{slow} are the first-order rate constants of the fast and slow phases of reaction, respectively. The data of the soluble enzyme show $A_{\text{fast}} \approx A_{\text{slow}} \approx 50\%$ of the initial activity.

However, the thermal inactivation for immobilized urease at 95°C was found to be monophasic, i.e., all of the activity was destroyed in a single phase. At this point, it could be assumed that at very high temperatures, the asymmetry in the urease molecule cannot be retained.

Estimation of Blood Urea with Immobilized Urease Beads

The chitosan beads with immobilized urease were subsequently used to assay the blood urea of some patients from the local clinical pathology laboratory. Conditions such as the number of beads and incubation period were worked out with 40 mg/dL of urea, which is much higher than the normal physiologic range (20–40 mg/dL). About three to four beads were found to bring about the complete hydrolysis of 400 mg/dL of urea in a

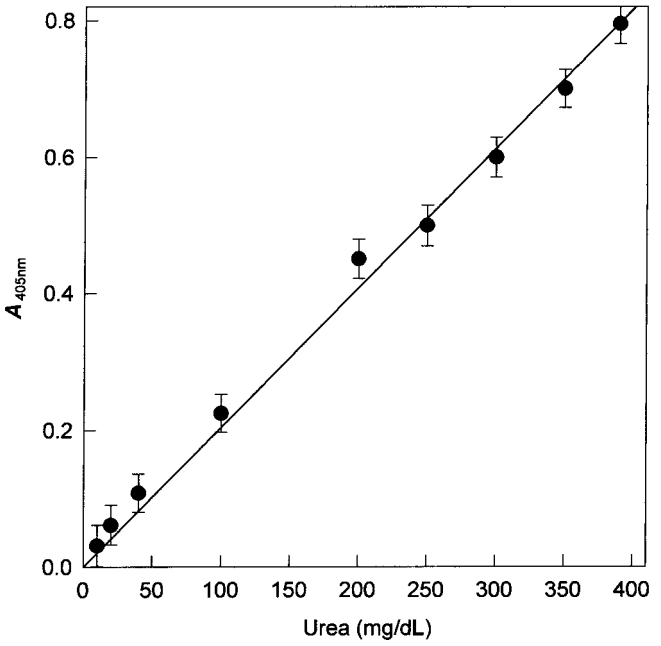

Fig. 6. Calibration curve for the urea assay using immobilized urease on chitosan beads (see Materials and Methods).

Table 2
Estimation of Blood Urea with Immobilized Urease

Chemical method (mg/dL)	Immobilized enzyme (mg/dL)[a]
36	35.5
23	21.1
20	19.0
123	121.5
21	21.0
21	21.5
26	25.0
31	30.5
24	25.0
33	34.0

[a]Values obtained are an average of three repeats.

20-min incubation (data not shown). A calibration curve with urea concentration ranging from 10 to 400 mg/dL is shown in Fig. 6.

The values for the urea concentration in the clinical blood samples determined with our immobilized urease compare favorably with those obtained by the clinical pathology laboratory (Table 2). Thus, urease bound

to chitosan beads may be valuable as an analytical tool for the estimation of urea in clinical laboratories.

Continuous Monitoring of Urea in Serum

The packed-bed reactor of the chitosan beads (12 nos) was used successfully for estimation of 10 serum urea samples over a period of 1 wk (data not shown).

There have been reports in this country on the commercialization of "synthetic milk," but this milk contained unacceptable concentrations of urea. Thus, in the future, to monitor the quality of milk, it will become necessary to screen all milk samples for urea content, and continuous methods like the one described here may find a potential application. Studies in this direction are in progress in our laboratory.

Acknowledgments

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