Journal of Molecular Catalysis B: Enzymatic 58 (2009) 138–145

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of soybean (*Glycine max*) urease on alginate and chitosan beads showing improved stability: Analytical applications

Sandeep Kumar[1], Alka Dwevedi, Arvind M. Kayastha*

School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi 221005, India

ARTICLE INFO

Article history:
Received 13 August 2008
Received in revised form
29 November 2008
Accepted 9 December 2008
Available online 24 December 2008

Keywords:
Immobilization
Soybean
Glycine max
Urease
Chitosan
Alginate

ABSTRACT

The soybean (*Glycine max*) urease was immobilized on alginate and chitosan beads and various parameters were optimized and compared. The best immobilization obtained were 77% and 54% for chitosan and alginate, respectively. A 2% chitosan solution (w/v) was used to form beads in 1N KOH. The beads were activated with 1% glutaraldehyde and 0.5 mg protein was immobilized per ml of chitosan gel for optimum results. The activation and coupling time were 6 h and 12 h, respectively. Further, alginate and soluble urease were mixed to form beads and final concentrations of alginate and protein in beads were 3.5% (w/v) and 0.5 mg/5 ml gel. From steady-state kinetics, the optimum temperature for urease was 65 °C (soluble), 75 °C (chitosan) and 80 °C (alginate). The activation energies were found to be 3.68 kcal mol^{-1}, 5.02 kcal mol^{-1}, 6.45 kcal mol^{-1} for the soluble, chitosan- and alginate-immobilized ureases, respectively. With time-dependent thermal inactivation studies, the immobilized urease showed improved stability at 75 °C and the $t_{1/2}$ of decay in urease activity was 12 min, 43 min and 58 min for soluble, alginate and chitosan, respectively. The optimum pH of urease was 7, 6.2 and 7.9 for soluble, alginate and chitosan, respectively. A significant change in K_m value was noticed for alginate-immobilized urease (5.88 mM), almost twice that of soluble urease (2.70 mM), while chitosan showed little change (3.92 mM). The values of V_{max} for alginate-, chitosan-immobilized ureases and soluble urease were 2.82×10^2 μmol NH_3 min^{-1} mg^{-1} protein, 2.65×10^2 μmol NH_3 min^{-1} mg^{-1} protein and 2.85×10^2 μmol NH_3 min^{-1} mg^{-1} protein, respectively. By contrast, reusability studies showed that chitosan–urease beads can be used almost 14 times with only 20% loss in original activity while alginate–urease beads lost 45% of activity after same number of uses. Immobilized urease showed improved stability when stored at 4 °C and $t_{1/2}$ of urease was found to be 19 days, 80 days and 121 days, respectively for soluble, alginate and chitosan ureases. The immobilized urease was used to estimate the blood urea in clinical samples. The results obtained with the immobilized urease were quite similar to those obtained with the autoanalyzer®. The immobilization studies have a potential role in haemodialysis machines.

1. Introduction

Enzyme immobilization has been a prerequisite for industries based on enzyme hydrolyzed products. It has also been successfully implemented at lab scale for enzyme stabilization from various environmental factors including pH, temperature, buffers, etc. and traces of unknown inhibitors. Several methods of enzyme immobilization have been known, each of them have their own limitations and benefits. Adsorption is simple, cheap and effective but frequently reversible, covalent attachment and cross-linking are effective and durable, but expensive and easily worsening the enzyme performance, and in membrane reactor-confinement, entrapment and micro-encapsulations diffusional problems are inherent. Consequently, as a rule the optimal immobilization conditions for a chosen enzyme and its application are found empirically by a process of trial and error in a way to ensure the highest possible retention of activity of the enzyme, its operational stability and durability [1–3]. Enzyme immobilizations onto matrices which are non-toxic, cheap, renewable, biodegradable have utmost importance in food, cosmetics, biomedical, or pharmaceuticals applications [4]. Biopolymers like alginate, chitin, chitosan, agarose, agar, etc. have been worthy matrices for enzyme immobilization [5].

Alginate is a water soluble linear polysaccharide extracted from brown seaweed and is composed of alternating blocks of 1 → 4-linked α-L-guluronic and β-D-mannuronic acid residues. The gelation of alginate can be carried out under an extremely mild

* Corresponding author. Tel.: +91 542 2368331; fax: +91 542 2368693.
E-mail address: kayasthabhu@gmail.com (A.M. Kayastha).
[1] Present address: Department of Biochemistry & Physiology, College of Biotechnology, Sardar Vallabh Bhai Patel University of Agriculture & Technology, Modipuram, Meerut 250110, India.

1381-1177/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2008.12.006

environment and uses non-toxic reactants. The most important property of alginates is their ability to form gels by reaction with divalent cations such as Ca^{2+}. Alginate beads can be prepared by extruding a solution of sodium alginate containing the desired protein, as droplets, into a divalent cross-linking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. The gelation and cross-linking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of these guluronic groups to form the characteristic egg-box structure. Amount of divalents affects the various properties of alginate including porosity, swelling behavior, stability, biodegradability, gel strength, viscoelasticity, etc. [6–9]. The calcium alginate gels are the most extensively studied. The ability of alginate to form two types of gel dependent on pH, i.e., an acid gel and an ionotropic gel, gives the polymer unique properties compared to neutral macromolecules. The physico-chemical properties of the polymer system and the swelling process are dependent on the type of gel formed [6,9].

Chitosan is the most important derivative obtained from chitin, a natural polysaccharide constitutive of crustaceous shells (shrimp, crab and other shellfish). Removal of most of the acetyl groups of chitin by treatment with strong alkalis yields chitosan [10]. Chitosan is linear copolymer polysaccharide consisting of β (1 → 4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) units. The term chitosan is used to describe a series of polymers of different degrees of deacetylation (DD), defined in terms of the percentage of primary amino groups in the polymer backbone, and average molecular masses (M_r). The DD of typical commercial chitosan is usually between 70% and 95%, and the M_r between 10 kDa and 1000 kDa. The properties, biodegradability and biological role of chitosan is frequently dependent on the relative proportions of *N*-acetyl-D-glucosamine and D-glucosamine residues [11]. The solubility of chitosan in solution depends on DD, the ionic concentration, the pH, the nature of the acid used for protonation and the distribution of the acetyl groups along the chain, as well as to the conditions of isolation and drying [1,5,11].

Soybean (*Glycine max*) has been economically very important to be used as source of various proteins for industrial purpose. Due to the fact that besides helpful for protein isolation (rich source), byproducts produced have much importance to poultry, fisheries, feed for dairy cattles, etc. It has been reported that it is very much beneficial to lactating dairy cows, pigs, and chicken due to its easy digestibility. Among various proteins present in soybean, it also contains urease (EC 3.5.1.5), which is present in abundance in its seeds. Ureases have been providing conceptual milestones throughout the history of modern biology and catalyze the conversion of urea to ammonia and carbon dioxide. This enzyme is present in two isoforms [12]; the embryo-specific urease, which is synthesized only in the developing embryos [13,14] and the ubiquitous urease, which is found in all vegetative tissues [15,16]. One role of the ubiquitous urease, in recycling metabolically derived urea, has been demonstrated in a number of experimental conditions [17–20]. In spite of the high concentration of the protein in the seeds, it has been suggested that the embryo-specific urease plays no role in nitrogen assimilation from urea [18,19,21].

The present work reports the immobilization of soybean urease isolated from the mature seeds onto alginate and chitosan. The physico-chemical properties of immobilized urease were compared with that of soluble form. To check the accuracy of our method, the ureolytic activity of immobilized urease were compared with the results of autoanalyzer®, frequently being used by pathological laboratories for multiple sample analysis.

2. Materials and methods

2.1. Chemicals and enzyme

Crab shell chitosan (with a degree of deacetylation of 75% and molecular mass of 13.4×10^5 kDa), sodium alginate (viscosity: ≥2000 cP, at 25 °C), urea (enzyme grade), Tris buffer, BSA and dialysis tubing were purchased from Sigma Chemicals Co., St. Louis, MO, USA. Glutaraldehyde was obtained from Fluka Biochemika, Germany. The Nessler's reagent (NR) and trichloroacetic acid (TCA) were purchased from HiMedia Laboratories, India. Serum samples were collected from a local pathology laboratory. All reagents were of analytical grade. All reagents were prepared in Milli Q water (Millipore, USA). Urease was isolated and purified from the mature seeds of soybean (*Glycine max*) to apparent homogeneity by the method of Polacco and Havir [13] with minor modifications.

2.2. Enzyme and protein assay

The appropriate amount of soluble or immobilized urease was incubated in 0.1 M urea with intermittent shaking. The amount of NH_3 liberated after incubation for a fixed time interval was determined using Nessler's reagent [22]. The absorbance was measured spectrophotometrically at 405 nm (ATI-UNICAM UV-Vis spectrophotometer, UK). One unit of urease activity liberates 1 μmol of NH_3 from 0.1 M urea per min at standard assay conditions (0.05 M Tris-acetate buffer, pH 7 and 37 °C).

The protein content of soluble and immobilized urease was estimated by the method of Lowry et al. [23] using BSA as standard. The amount of protein immobilized on chitosan beads was determined by subtracting the residual protein (protein left in urease solution after incubation + proteins in washings) from the total protein (enzyme solution used for incubation of chitosan beads).

2.3. Assay of blood urea with immobilized urease

The immobilized urease (2–3 beads) was incubated in 0.8 ml of 0.05 M Tris-acetate buffer (pH 7.0) for 5 min at 37 °C. The reaction was started by adding 0.2 ml of serum. After 20 min, 0.5 ml of the reaction mixture was taken out and reacted with 0.5 ml of 10% (w/v) trichloroacetic acid. The precipitated proteins were removed by centrifugation and the urea was estimated in the supernatant as described above. The urea was estimated using the standard plot prepared with the known urea concentrations.

2.4. Immobilization of urease

2.4.1. Preparation of chitosan beads

The different concentrations (1.0–3.0%, w/v) of chitosan were used for preparing beads and the most appropriate concentration was used for subsequent studies. The stock solution of chitosan was prepared in Milli Q water, with 1.5% (v/v) acetic acid and heating to 60–70 °C. A syringe was clamped at a height of 15 cm above 1N KOH solution (continuously stirred at 37 °C) and its nozzle was closed with a piece of parafilm. The solution was added drop wise extruding through a hole (dia. 1 mm) in the parafilm. The beads of dia. 2.5 mm and uniform shape obtained were immediately washed with Milli Q water and stored in water at 4 °C till activated by glutaraldehyde.

2.4.2. Immobilization on chitosan beads

The chitosan beads were incubated with different concentrations of glutaraldehyde, in the range 0.5–5.0% (v/v) for 8 h at 37 °C. After incubation, the beads were washed properly with 0.1 M Tris-acetate buffer, pH 7.6 and stored in the same buffer till further use. The activated beads were incubated with varying concentrations of

urease preparation for 20 h at 4 °C. The beads were washed with 0.1 M Tris-acetate buffer, pH 7.6 to remove unbound urease. The activity and protein content of chitosan beads were determined. To determine the optimum time required for their activation, the beads were kept in 1% glutaraldehyde for different time intervals. Furthermore, the activated beads were incubated in desired concentration of urease solution for different time intervals to determine the coupling time. The percent immobilization (percent enzyme activity retention) was calculated as follows:

$$\text{percent immobilization}\,(\%) = \frac{\text{specific activity of immobilized urease}}{\text{specific activity of soluble urease}} \times 100$$

Specific activity of immobilized urease was determined by subtracting specific activity of urease during washing (unbound urease) from specific activity of soluble enzyme.

2.4.3. Immobilization on alginate beads

The stock solution of sodium alginate (5.5%, w/v) was prepared in 0.1 M Tris-acetate buffer, pH 7.6 at 70 °C with continuous stirring. It was allowed to cool to 4 °C and mixed with appropriately diluted urease (fixed protein concentration). The concentration of alginate was varied over the range, 1–5% (w/v) while keeping the concentration of urease fixed. The syringe was clamped at a height of 15 cm above and solution was dropped in same way as described for chitosan. The mixture was added drop wise into 8% (w/v) $CaCl_2$ solution (stirred continuously at 4 °C) to obtain beads of uniform size and shape. The alginate beads (dia. 2.5 mm) were formed and left for stirring for another 2–3 h to allow them to attain rigidity. Finally, the beads were washed with 0.1 M Tris-acetate buffer, pH 7.6 to remove the traces of $CaCl_2$ and stored in same buffer at 4 °C. The percent immobilization was determined similarly as described in case of chitosan beads.

2.5. Steady-state kinetics

The effect of pH on enzyme activity (soluble as well as immobilized) was investigated in pH range, 4.6–9.5. Three types of buffers were used for this study: citrate phosphate buffer (pH 4.6–6.6), phosphate buffer (pH 6.6–7.6) and Tris-acetate buffer (pH 7.2–9.5). The effect of temperature ranging from 20 °C to 85 °C was studied by assaying at similar conditions as described in section on enzyme assay. Effect of substrate concentration on urease activity was investigated at 37 °C by varying the urea concentration from 5 mM to 20 mM at optimum pH for soluble and immobilized enzyme. The activity assay was performed as described earlier and K_m was determined by Lineweaver–Burk plot.

2.6. Stability studies

2.6.1. Reusability

The immobilized urease was tested for its reusability by checking the activity using assay as described in section on enzyme assay at different time intervals. After every use, the beads were washed properly with 0.1 M Tris-acetate buffer, pH 7.6 and stored in the same buffer at 4 °C, till further use.

2.6.2. Thermal inactivation

The soluble and immobilized urease was incubated at 75 °C in water bath for a fixed time intervals. The enzyme (soluble and immobilized) was taken out at specified time intervals, chilled immediately and transferred to assay mixture. The activity assay was performed as described earlier and the residual activity was determined. The $t_{1/2}$ values for soluble and immobilized urease were determined.

2.6.3. Storage stability

The soluble and immobilized ureases were stored in 0.1 M Tris-acetate buffer, pH 7.6 at 4 °C. The activity was determined and recorded at regular intervals for stored urease (immobilized and soluble) using assay procedures described in section on enzyme assay under similar conditions. The semi-log values of percent residual activity were plotted against the number of days. The $t_{1/2}$ of soluble and immobilized urease was calculated from such a plot.

3. Results and discussion

3.1. Optimum conditions for urease immobilization

Generally the entrapment and adsorption methods are less efficient as compared to covalent methods, but these methods are comparatively easier to perform, and are less harsh to enzyme besides, being inexpensive. These methods can find applications in medicines, agriculture and analytical tools, where they can replace chemicals, which might be toxic and harsh to living systems. Covalent immobilization has been worked out with various kinds of supporting matrices (mostly polysaccharides and other organic polymers) following several different activation and coupling procedures.

During preparation of alginate beads, the beads were soft and flaccid at lower concentrations of the alginate, while there was low percent immobilization at higher concentrations (Table 1). The diffusional resistances offered by alginate gel at higher concentration to substrate might be responsible for decreased hydrolysis of urea and therefore percent immobilization was affected. It was observed that with 3.5% alginate (w/v), rounded and uniformly shaped beads (dia. 2.5 mm) were formed and yielded 34.7% immobilization. From storage studies, increased rate of urease leaching was observed at lower concentrations of alginate (results not shown). Therefore, the concentration of sodium alginate was fixed at 3.5% for subsequent experiments. Protein concentration was varied and the best immobilization observed was 54% at 0.5 mg protein/5 ml alginate gel. At higher protein concentrations, the immobilization efficiency was reduced.

While standardizing conditions for urease immobilization on chitosan, the volume of enzyme solution (0.5 ml) and number of beads incubated (four) were kept constant in all the experiments. With chitosan at lower concentrations, the beads were fragile, discoid and soft while at higher values, the solution was very viscous and thick, and therefore was difficult to cast. A 2% (w/v) solution of chitosan was suitable for preparing firm, hard and spherical beads (data not shown). The glutaraldehyde treatment at higher

Table 1
Standardizing the various conditions for urease immobilization in alginate beads.

Concentration of sodium alginate % (w/v)	Protein/5 ml of alginate gel (mg)	Immobilization[a] (%)
1.0	0.838	No beads
2.0	0.838	26.74 ± 1.34
3.0	0.838	32.66 ± 1.03
3.5	0.838	34.66 ± 1.13
4.0	0.838	31.67 ± 1.15
5.0	0.838	30.09 ± 1.26
3.5	0.165	44.27 ± 1.29
3.5	0.335	51.19 ± 1.15
3.5	0.500	**54.04 ± 1.12**
3.5	0.670	51.93 ± 1.22
3.5	0.950	43.57 ± 1.25
3.5	1.005	43.06 ± 1.14

Bold values correspond to maximum % immobilization corresponding to the conditions specified.

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

Table 2
Standardizing the various parameters for the immobilization of urease on chitosan beads.

Glutaraldehyde conc. (%)	Protein/ml of chitosan gel (mg)	Activation time (h)	Coupling time (h)	Immobilization[a] (%)
Variation of glutaraldehyde concentration				
0.5	0.2	8	20	58.5 ± 1.2
1.0	0.2	8	20	**65.3 ± 1.0**
2.0	0.2	8	20	63.1 ± 0.8
3.0	0.2	8	20	61.3 ± 0.9
5.0	0.2	8	20	51.3 ± 0.9
Variation of protein concentration				
1.0	0.2	8	20	63.0 ± 1.0
1.0	0.3	8	20	71.0 ± 1.4
1.0	0.4	8	20	75.0 ± 0.9
1.0	0.5	8	20	**77.0 ± 1.5**
1.0	0.6	8	20	76.1 ± 0.8
1.0	0.7	8	20	76.3 ± 1.3
Variation of activation time				
1.0	0.5	5	20	71.0 ± 0.9
1.0	0.5	6	20	**75.0 ± 1.2**
1.0	0.5	12	20	74.0 ± 1.0
1.0	0.5	20	20	73.9 ± 1.4
Variation of coupling time				
1.0	0.5	6	3	52.9 ± 1.1
1.0	0.5	6	6	61.0 ± 0.9
1.0	0.5	6	12	**76.0 ± 1.1**
1.0	0.5	6	24	76.1 ± 1.0

Bold values correspond to maximum % immobilization corresponding to the conditions specified.
[a] Values obtained are an average of three repeats.

concentrations made the beads brittle, fragile and the efficiency of immobilization was also affected (Table 2). Glutaraldehyde 1% (v/v) was the optimal concentration for activation of beads. The beads become brownish in color after glutaraldehyde treatment. The efficiency of immobilization was 77%, when all the parameters were optimized, suggesting that there was decrease in specific activity of urease on immobilization (see equation on percent immobilization in Section 2). Kayastha et al. [24] have also reported a decrease in specific activity of enzyme in immobilized state than that in soluble state. The protein concentration used for incubating beads was varied from 0.2 mg/ml to 0.7 mg/ml with 0.5 mg/ml of chitosan gel gave the best results. Obtained immobilization was much higher than that of urease from pigeonpea immobilized onto chitosan beads [25] with about twice the amount of enzyme was immobilized. Higher protein concentrations did not yield better immobilization, while the lower concentrations were not enough to saturate most of the enzyme binding sites on the activated matrix. The chitosan beads were investigated further to find out the activation time. The incubation periods were varied from 3 h to 20 h and it was observed that 6 h incubation was most appropriate. Furthermore, the coupling time was also optimized at 12 h by incubating the beads in urease solution for different time intervals.

Earlier from this laboratory ureases from some plant sources were immobilized on different matrices with varying efficiencies, viz. on gelatin via glutaraldehyde, 67.6% [26]; on agar by entrapment, 51.7% [27]; on gelatin via glutaraldehyde, 75% [28]; on polyacrylamide, 50% [29]; on DEAE-cellulose paper, 51% [30]; flannel cloth via polyethyleneimine, 56% [31]. In addition, there are many reports of urease immobilization on various hydrogels by means of different methods, e.g., on poly(acrylonitrile)chitosan composite membranes [32]; on chitosan–alginate polyelectrolyte complexes (C–A PEC) and poly(acrylamide-*co*-acrylic acid)/kappa-carrageenan (P(AAm-*co*-AA)/carrageenan) hydrogels [33]; encapsulation within kappa-carrageenan beads [34]; within polyanionic carboxymethylcellulose/alginate (CMC/Alg) microspheres coated with a cationic polysaccharide, chitosan (C) [35].

3.2. Steady-state kinetics

3.2.1. Effect of pH

The effect of pH on the activity of free and immobilized urease is shown in Fig. 1. The optimum pH for soluble and alginate-immobilized urease was 7 and 6.2, respectively. From the data, a shift in the pH optima can be noticed which is towards the acidic side, displaced through 0.8 units. Urease is present inside alginate beads, due to which substrate (urea) and product (NH_3) suffer from diffusional restraints. Released NH_3 inside the alginate bead, turns the microenvironment of enzyme little basic than that present in the bulk phase. Therefore, enzyme works at optimum pH even though the pH of bulk phase which was acidic. On the other hand, the optimum pH value for chitosan-immobilized urease was 7.9, the shift was on opposite side (basic side) through 0.9 units. In case of chitosan-immobilized urease, enzyme is present at the surface of chitosan beads due to which released product (NH_3) suffers

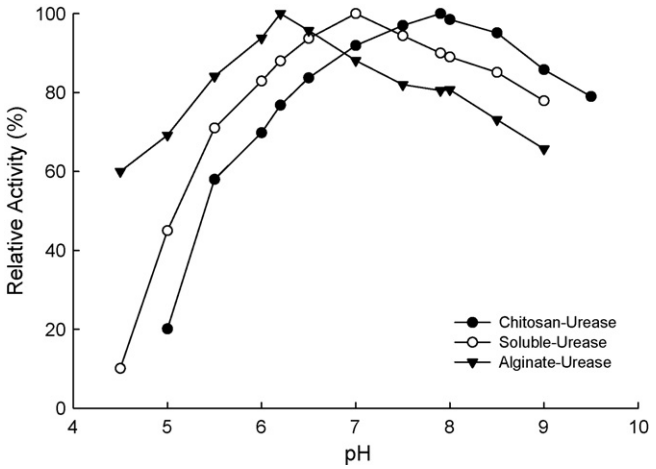

Fig. 1. The effect of pH on the activity of soluble and immobilized urease. Each experimental point represents the mean of three determinations.

no diffusional restraints. Ammonia (NH_3) gets dissolved immediately in the solution and forms NH_4^+ leading to lowering of pH of the solution. Furthermore, decrease in positive charge density of chitosan at alkaline condition (pK_a of chitosan is ~6.5) favors maintenance of lower pH in the enzyme's microenvironment leading to shift in optimum pH towards alkaline side. Krajewska [36] and Krajewska and Piwowarska [37] have also discussed significant role of microenvironment on the physico-chemical properties of immobilized enzyme.

Kayastha and Srivastava [25] reported the similar kind of shift (pH 7.3–8.5) when the pigeonpea urease was immobilized covalently on chitosan. The jack bean urease immobilized on a fixed-bed reactor showed the displacement in pH from 6.6 to 7.6 [38], however, when immobilized on the porous glass beads and molecular sieve 4A, the shift was reported toward acidic side [39,40]. From earlier reports on immobilized pigeonpea urease, shifts in pH optima have been observed [26,27]. In general, 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 when immobilized on a solid matrix. Depending on the surface and residual charges on the solid matrix and the nature of the bound enzyme, 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 [41].

3.2.2. Effect of temperature

The stability and kinetics of soluble and immobilized urease was studied at different temperatures (Fig. 2). The optimum temperature for soluble urease was found to be 65 °C. The optimum temperature for chitosan-immobilized urease and alginate-immobilized urease were found to be 75 °C and 80 °C, respectively. Bissett and Sternberg [42] have reported similar results when *Aspergillus* β-glucosidase was immobilized on chitosan. Similar reports on increase in optimum temperature during enzyme immobilization have been previously reported [27,28,30,33]. The data (temperature range 20–60 °C) from Fig. 2 was reploted as Arrhenius plot (Fig. 3) and activation energies were found to be 3.68 kcal mol^{-1}, 5.02 kcal mol^{-1}, 6.45 kcal mol^{-1} for soluble urease and chitosan- and alginate-immobilized ureases, respectively. The change in activation energy after immobilization of urease has also been reported earlier [42,43]. Clearly at high temperatures, loss in the urease activity of soluble form was due to denaturation of enzyme. The improved stability of immobilized enzyme was supposed to be aided by the matrix, which absorbs a considerable amount of heat and protects the enzyme against denaturation. In

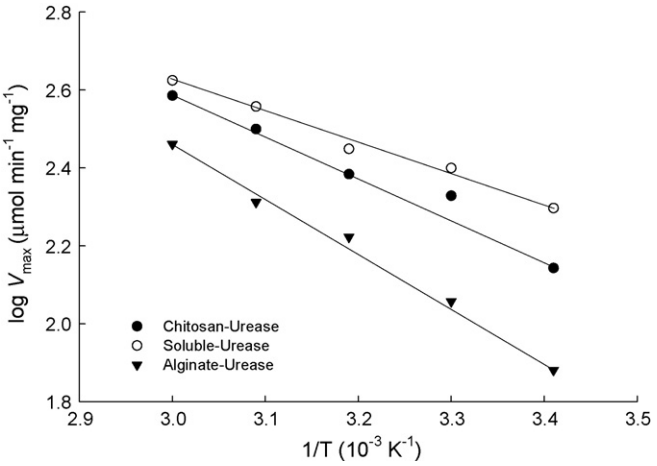

Fig. 3. Arrhenius plot from data in Fig. 2. Each experimental point represents the mean of three determinations.

industries, most of the operations are carried out either at room or higher temperatures. Usually, the immobilization improves enzyme stability against the higher temperatures and therefore makes the enzyme industrially more useful and economical.

3.2.3. Effect of substrate concentration

The diffusion of urea through the alginate matrix appears to exert the major influence on the observed kinetics. The matrix often prevents free diffusion of substrate and therefore results in changed K_m. The soybean urease was immobilized in alginate gel by entrapment method and investigated for the effect of substrate concentration on urease activity. It was observed that there was a significant increase in K_m for alginate-immobilized urease (5.88 mM) when compared with soluble urease (2.70 mM), which is due to diffusional resistance offered by the gel. The urea takes more time to reach the site of catalysis and therefore results in an increase in K_m. Similarly, the chitosan immobilized urease showed an apparent K_m value of 3.92 mM, which was about 1.22 mM units higher than soluble urease (Fig. 4). 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

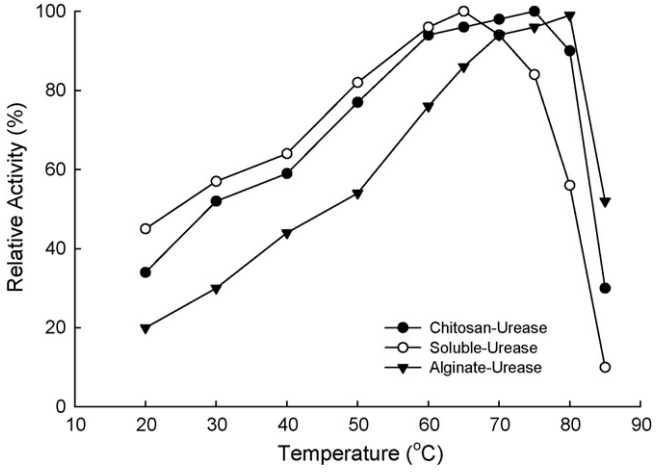

Fig. 2. The effect of temperature on the activity of soluble and immobilized urease. Each experimental point represents the mean of three determinations.

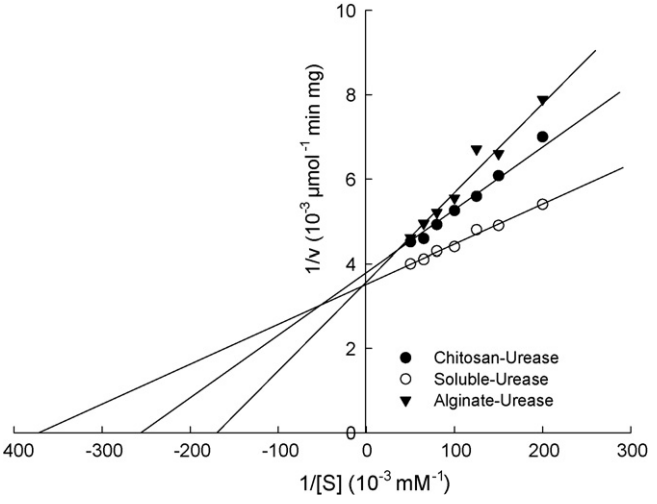

Fig. 4. Determination of K_m for immobilized urease by Lineweaver–Burk plot method. Each experimental point represents the mean of three determinations.

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. Similar changes in K_m have also been reported for urease when immobilized by encapsulation and adsorption methods on various matrices like, chitosan, gelatin, DEAE-cellulose paper, etc. [25,28,30]. The V_{max} of alginate-immobilized urease, chitosan-immobilized urease and soluble urease were 2.82×10^2 μmol NH_3 min^{-1} mg^{-1} protein, 2.65×10^2 μmol NH_3 min^{-1} mg^{-1} protein and 2.85×10^2 μmol NH_3 min^{-1} mg^{-1} protein, respectively (Fig. 4). As observed, chitosan-immobilized urease has lower V_{max} with respect to soluble and alginate urease. It is due to the fact that enzyme was covalently attached to chitosan using glutaraldehyde and during this, some of the enzyme molecules get attached via groups of active site leading to their inactivation, thus a decrease in V_{max} was observed [42,43]. Furthermore, according to comparative analysis of V_{max} of soluble and immobilized enzyme, a decrease of about only 10% was observed. This was due to increase in local enzyme concentration during immobilization as previously reported [24].

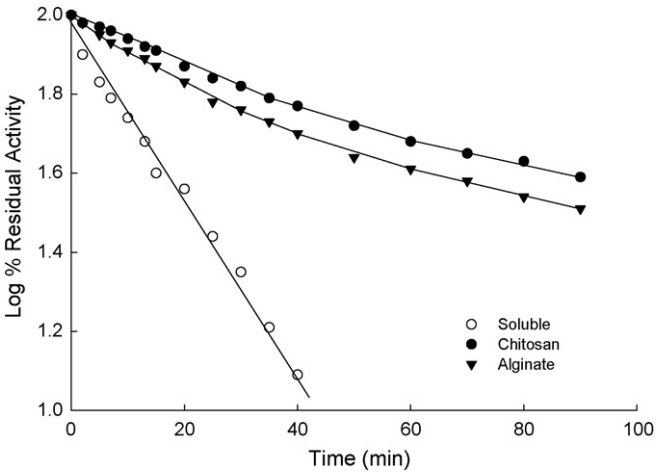

Fig. 6. Time-dependent thermal inactivation studies with soluble and immobilized urease. Each experimental point represents the mean of three determinations.

3.3. Stability studies

For industrial use of enzymes, it is important to consider the enzyme stability with respect to various parameters like temperature, reusability, storage stability, etc. The soluble enzyme cannot be recovered from the reaction mixtures and therefore cannot be used to catalyze more reactions, but the process of immobilization can make it feasible. The urease was investigated for the reusability studies. The studies revealed that the chitosan-immobilized urease after 14 uses retained almost 80% of the original activity and the alginate-immobilized urease retained only 54% (Fig. 5). Also it was noticed that the alginate beads became soft and clumsy after 7–8 uses and it becomes difficult to use in the subsequent reactions. Furthermore, chitosan beads were also tested for their stability for reaction mixture and it was found that they were quite stable with respect to their texture/shape, etc.

The soluble as well as the immobilized urease were incubated at 75 °C for the indicated time intervals and the data collected were analyzed for thermal stability (Fig. 6). When the log % residual activity retained was plotted against time, at 75 °C, the free enzyme showed first-order reaction however, the immobilized enzyme follows non-linear relation. Evidently, when compared with that of soluble urease, the rate of decay in the activity at 75 °C (elevated temperatures) with time was significantly reduced after immobilization. Although, the rate was not linear for immobilized urease, but was almost in single phase. It clearly establishes the improved stability of immobilized urease against elevated temperatures after incubation for longer durations. A similar situation has been observed for immobilized glucose isomerase [44]. A possible explanation for the observed non-linear relation for activity decay, might be that the number of cross-links to the support, to other enzyme molecules, and within a single enzyme molecule varied, resulting in heterogeneity in the thermal stability of the enzyme [42]. The $t_{1/2}$ for the decay was about 12 min, 43 min and 58 min for the free, alginate-immobilized and chitosan-immobilized enzymes, respectively.

For storage stability studies (Fig. 7), the immobilized urease was always kept in 0.1 M Tris-acetate buffers, pH 7.6 at 4 °C. All forms of urease were assayed on different days over a period of 180 days. The semi-log plot of % residual activity versus the number of days gave a $t_{1/2}$ of 19 days for soluble urease; 80 days for alginate-immobilized urease and 121 days for chitosan-immobilized urease, respectively. The chitosan-immobilized urease was found to be active for longer durations. From earlier reports, storage stability ($t_{1/2}$) of immobilized pigeon pea urease on agar, gelatin, alginate and chitosan were, respectively, 53, 75, 110 and 240 at 4 °C [25,27–29]. The alginate and chitosan beads were checked for urease leaching during the first 25 days of the storage stability period and practically no leaching was observed for chitosan-immobilized urease, but alginate-immobilized urease showed leaching during this period.

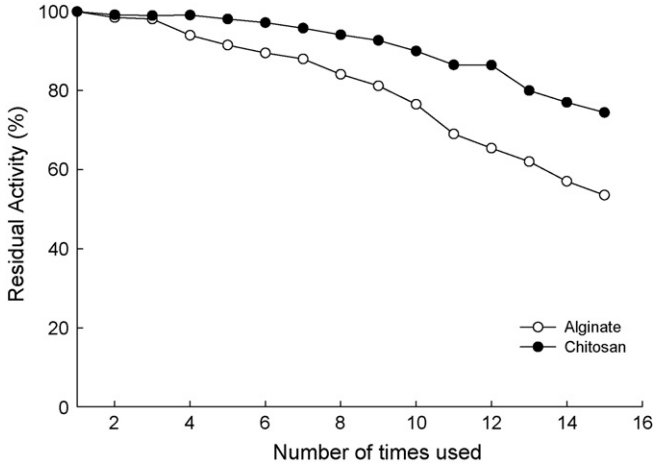

Fig. 5. Reusability studies with immobilized urease. Each experimental point represents the mean of three determinations.

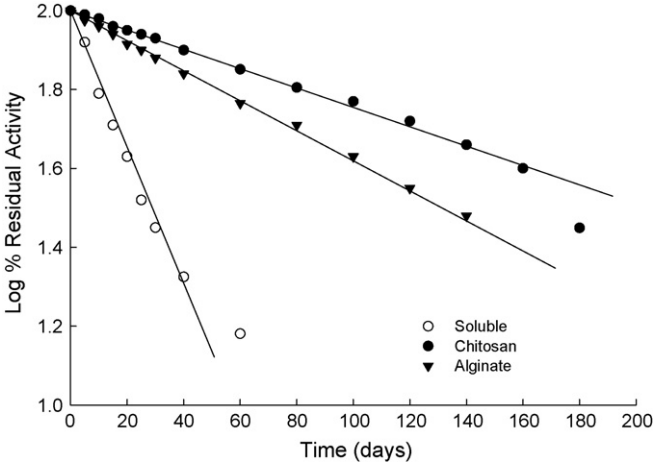

Fig. 7. Storage stability of soluble and immobilized urease at 4 °C. Each experimental point represents the mean of three determinations.

Table 3
Estimation of blood urea by immobilized urease compared with the data from Autoanalyzer®.

Sample number	Calcium alginate beads[a] (mg/dl)	Chitosan beads[†] (mg/dl)	Autoanalyzer® (mg/dl)
1.	27 ± 0.6	28 ± 0.7	29
2.	31 ± 0.2	31 ± 0.5	32
3.	38 ± 0.3	39 ± 0.4	40
4.	56 ± 0.4	55 ± 0.8	57
5.	19 ± 0.5	19 ± 0.9	20
6.	81 ± 0.2	81 ± 0.5	82
7.	69 ± 0.7	70 ± 0.2	71
8.	34 ± 0.5	34 ± 0.2	35
9.	29 ± 0.5	29 ± 0.3	30
10.	46 ± 0.7	47 ± 0.5	48
11.	34 ± 0.8	35 ± 0.6	36
12.	21 ± 0.5	22 ± 0.2	23

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

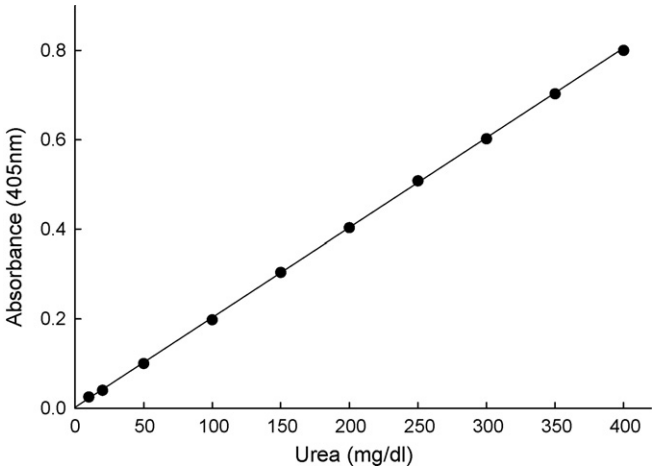

Fig. 8. Calibration plot for the estimation of serum urea using immobilized urease. Each experimental point represents the mean of three determinations.

3.4. Estimation of serum urea with immobilized urease

In order to determine the accuracy of urea estimation by immobilized urease (chitosan–urease and alginate–urease), the values estimated for urea present in the same serum samples using autoanalyzer® (Autoanalyzer FT-2; Zydus Co., Rome) by a local clinical pathology laboratory were used for comparison. The beads (2–3) were subsequently used to assay the blood urea samples. Various conditions such as number of beads, incubation period, etc., were worked out using 400 mg of urea/dl, which is much higher than the normal physiological range (20–40 mg/dl). Approximately 2–3 beads were found to bring about the complete hydrolysis of 400 mg of urea/dl in 20 min. A calibration curve with urea concentration ranging from 10 mg/dl to 400 mg/dl is shown in Fig. 8. The urea assay of serum samples is given in Table 3. The values for the urea concentration in the clinical blood samples determined with the immobilized urease compare favorably with those obtained by the autoanalyzer® being frequently used by clinical pathology laboratories. The easy availability of the soybean urease, the ease of its immobilization on hydrogel like calcium alginate and chitosan and a significantly lower cost of the urease makes it a suitable product for future applications in therapeutics and diagnostics.

4. Conclusions

The chitosan and alginate were successfully used as support material for immobilizing urease. The optimum immobilization was quite satisfactory with the matrices viz., 77% with chitosan and 54% with alginate. The rate of mass transfer of the substrate and products to and from immobilized enzymes presents problems, which are not among free enzymes. For the substrate to be acted upon by an immobilized enzyme it must diffuse into the matrix and the products must diffuse out. These diffusional processes often result in a lower concentration of substrate and a higher concentration of product at the enzyme site than in the bulk solution. All these factors affect the physico-chemical properties of urease. The excellent storage stability, thermal stability, reusability and operational stability of the immobilized urease demonstrates the superior potential of immobilized forms for various potential applications. The alginate and chitosan have many desirable characteristics, viz. hydrophilicity, biodegradability, biocompatibility and non-toxicity. Therefore, these hydrogels can be successfully used for preparing products, which are compatible with the living systems. Such urease-immobilized hydrogels can find applications in industries like biotechnology, biomedicine, agriculture and environment where they can be used in the construction of artificial kidney machines; the analysis of adulterated milk/contaminated water and the estimation of blood urea.

Acknowledgements

Facilities provided by School of Biotechnology are gratefully acknowledged. S.K. and A.D. are thankful to Council for Scientific and Industrial Research, New Delhi, India for financial support in the form of Junior and Senior Research Fellowships.

References

[1] B. Krajewska, Enzyme Microb. Technol. 35 (2004) 126.
[2] J.K. Park, H.N. Chang, Biotechnol. Adv. 18 (2000) 303.
[3] R.A. Sheldon, Adv. Synth. Catal. 349 (2007) 1289.
[4] A.S. Hoffman, Adv. Drug Deliv. Rev. 43 (2002) 3.
[5] M. Rinaudo, Polym. Int. 57 (2008) 397.
[6] M. George, T.E. Abraham, J. Control. Release 114 (2006) 1.
[7] H.H. Tønnesen, J. Karlsen, Drug Dev. Ind. Pharm. 28 (2002) 621.
[8] W.R. Gombotz, S.F. Wee, Adv. Drug Deliv. Rev. 31 (1998) 267.
[9] A.D. Augst, H.J. Kong, D.J. Mooney, Macromol. Biosci. 6 (2006) 623.
[10] Q.P. Peniston, E.L. Johnson, Process for the Manufacture of Chitosan, US Patent 4,195,175, in: G.A.F. Roberts (Ed.), 1992, Chitin Chemistry (Preparation of Chitin and Chitosan), Macmillan Press, p. 54 (1980).
[11] M. Rinaudo, Prog. Polym. Sci. 31 (2006) 603.
[12] M.A. Holland, J.D. Griffin, L.E. Meyer-Bothling, J.C. Polacco, Dev. Genet. 8 (1987) 375.
[13] J.C. Polacco, E.J. Havir, J. Biol. Chem. 254 (1979) 1707.
[14] R.S. Torisky, J.D. Griffin, R.L. Yenofsky, J.C. Polacco, Mol. Gen. Genet. 242 (1994) 404.
[15] J.C. Polacco, R.G. Winkler, Plant Physiol. 74 (1984) 800.
[16] J.C. Polacco, R.W. Krueger, R.G. Winkler, Plant Physiol. 79 (1985) 794.
[17] A. Goldraij, L.J. Beamer, J.C. Polacco, Plant Physiol. 132 (2003) 1801.
[18] J.C. Polacco, M.A. Holland, Int. Rev. Cytol. 145 (1993) 65.
[19] A. Sirko, R. Brodzik, Acta Biochim. Pol. 47 (2000) 1189.
[20] C.P. Witte, S.A. Tiller, M.A. Taylor, H.V. Davies, Plant Physiol. 128 (2002) 1129.
[21] N. Stebbins, M.A. Holland, S.R. Cianzio, J.C. Polacco, Plant Physiol. 97 (1991) 1004.
[22] N. Das, A.M. Kayastha, P.K. Srivastava, Phytochemistry 61 (2002) 513.

[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[24] A.M. Kayastha, P.K. Srivastava, B. Miksa, S. Slomkowski, J. Bioact. Compat. Polym. 18 (2003) 113.
[25] A.M. Kayastha, P.K. Srivastava, Appl. Biochem. Biotechnol. 96 (2001) 41.
[26] S. Kumar, A. Kansal, A.M. Kayastha, Orient. Pharm. Exp. Med. 5 (2005) 43.
[27] M. Swati, S. Kumar, K.R.S. Reddy, A.M. Kayastha, Appl. Biochem. Biotechnol. 142 (2007) 291.
[28] P.K. Srivastava, A.M. Kayastha, Srinivasan, Biotechnol. Appl. Biochem. 34 (2001) 55.
[29] N. Das, A.M. Kayastha, O.P. Malhotra, Biotechnol. Appl. Biochem. 27 (1998) 25.
[30] K.R.C. Reddy, P.K. Srivastava, P.M. Dey, A.M. Kayastha, Biotechnol. Appl. Biochem. 39 (2004) 323.
[31] N. Das, A.M. Kayastha, World J. Microbiol. Biotechnol. 14 (1998) 927.
[32] K. Gabrovska, A. Georgieva, T. Godjevargova, O. Stoilova, N. Manolova, J. Biotechnol. 129 (2007) 674.
[33] F. Kara, G. Demirel, H. Tümtürk, Bioprocess. Biosyst. Eng. 29 (2006) 207.
[34] S.H. Baysal, R. Karagöz, Prep. Biochem. Biotechnol. 35 (2005) 135.
[35] A.E. Elçin, Y.M. Elçin, Artif. Cells Blood Sub. Immob. Biotechnol. 28 (2000) 95.
[36] B. Krajewska, J. Bioact. Compat. Polym. 15 (2000) 155.
[37] B. Krajewska, Z. Piwowarska, Biocatal. Biotrans. 23 (2005) 225.
[38] H.J. Moynihan, C.K. Lee, W. Clarck, N.H.L. Wang, Biotechnol. Bioeng. 34 (1989) 951.
[39] L. Iyengar, P. Bajpai, A.V.S.P. Rao, Indian J. Biochem. Biophys. 19 (1982) 130.
[40] H.H. Weetall, L.S. Hersh, Biochim. Biophys. Acta 185 (1969) 464.
[41] K. Mosbach, Sci. Am. 224 (1971) 26.
[42] F. Bissett, D. Sternberg, Appl. Environ. Microbiol. 35 (1978) 750.
[43] P.A. Dickensheets, L.F. Chen, G.T. Tsao, Biotechnol. Bioeng. 19 (1977) 365.
[44] N.B. Havewala, W.H. Pitcher Jr., in: E.K. Pye, L.B. Wingard (Eds.), Enzyme Engineering, vol. II, Plenum Press, New York, 1974, p. 315.