Appl Biochem Biotechnol (2007) 142:291–297
DOI 10.1007/s12010-007-0022-7

Immobilization of Urease from Pigeonpea (*Cajanus cajan*) on Agar Tablets and Its Application in Urea Assay

Swati Mulagalapalli · Sandeep Kumar · Ravi Charan Reddy Kalathur · Arvind M. Kayastha

Received: 26 July 2006 / Accepted: 2 January 2007 /
Published online: 17 April 2007

Abstract The pigeonpea urease was immobilized on agar, a common gelling substance. The tablet strips were used as moulds to cast agar tablets of uniform shape and size. The time and temperature of solidification of agar was 6 min and 44 °C, respectively. The 5 % agar (*w*/*v*) and 0.019 mg protein/agar tablet yielded an optimum immobilization of 51.7%. The optimum pH was shifted through 0.2 U (from 7.3 to 7.5) towards basic side upon immobilization. The optimum temperature of soluble and immobilized urease was 30 °C and 60 °C, respectively, showing the improvement in thermal stability of urease. There was an increase in K_m from 3.23 to 5.07 mM after immobilization. The half-lives of soluble and immobilized urease were 21 and 53 days, respectively, at pH 7.3 and 4 °C. The urea was estimated in different blood samples with the help of immobilized urease and the results were consistent with those from clinical pathology laboratory through an autoanalyzer® (Zydus Co., Rome, Italy).

Keywords Immobilization · Urease · *Cajanus cajan* · Agar · Urea

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. The use of enzyme for analytical purpose is increasingly gaining importance due to some intrinsic advantage of the immobilized system [1]. Chemical methods of immobilization are generally better and stable as they involve the formation of covalent bond and thus little or no leaching is expected.

Urease appears to be found in most, if not in all plants [2] and is an abundant seed protein in many members of Fabaceae (Leguminaceae), Cucurbitaceae, Asteraceae, and Pinaceae [3]. All plants [4] and bacterial [5] urease are probably nickel metalloenzymes. Seed urease from jack bean was the first example of a natural nickel metalloenzyme [6].

S. Mulagalapalli · S. Kumar · R. C. R. Kalathur · A. M. Kayastha (✉)
School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi 221005, India
e-mail: kayasthabhu@gmail.com

Urease, which catalyses the hydrolysis of urea to ammonia and carbon dioxide, has been used in immobilized form in kidney machine for detoxification of blood [7, 8]. Immobilization of urease has been carried out on several matrices for analytical/clinical applications [9–14, 31].

Agar (more correctly agar–agar) is a dried hydrophilic colloid extracted from the cell wall of certain algae of the class Rhodophyceae. It is a polysaccharide that consists of agarose, which has a strong gelling ability. It finds applications as gelling agent in the food and in the pharmaceutical industry. It is reasonably acid stable compared to other polysaccharides and does not show any protein reactivity. The cost of this material, compared with the other materials commonly used for immobilization, is low.

Urease from pigeonpea has been purified, characterized, and used in the fabrication of urea biosensor by utilizing immobilized urease from the laboratory [9–16, 17]. The low cost and easy availability of the pigeonpea urease and simple procedure of its immobilization on agar–agar described in the present study makes it a suitable product for future applications in diagnostics and therapeutics. Our main objective was to develop an economical method of urea estimation in aqueous samples. Assay of blood urea is of great clinical importance particularly in the case of kidney malfunction [18–21].

Materials and Methods

Enzyme

Urease (EC 3.5.1.5) was isolated from the seeds of pigeonpea (*Cajanas cajan* L.) procured from the local market, and was purified as described previously [15].

Chemicals

Agar and Nesslers's Reagent (NR) were from HiMedia Laboratories, Mumbai, India. Urea (enzyme grade) and BSA were from Sigma. Tris and trichloroacetic acid (TCA) were from Sisco Research Labs, Mumbai, India. All reagents were of analytic grade either from Merck or BDH, India. All reagents were prepared in Milli Q (Millipore, USA) water.

Enzyme Assay

For soluble urease, the enzyme solution was appropriately diluted before the activity assay. The soluble/immobilized urease was incubated with 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; 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 urea per minute at pH 7.3 and 37 °C.

Protein Estimation

Protein was assayed by the method of Lowry et al. [22] using BSA as standard.

Immobilization of Urease

Tablet strips were preferred for the purpose of casting agar, as it could be easily poured into the tablet strips to obtain the agar tablets of uniform shape and size. The various

concentrations of agar (0.5 to 10% *w*/*v*) and protein (0.010 to 0.025 mg/agar tablet) were used to optimize the percent immobilization. For immobilization, the molten agar and soluble urease were mixed and immediately poured into moulds before the time of solidification. Care was taken to prevent the formation of air bubbles. The agar tablets were removed and stored in 0.025 M Tris–acetate buffer, pH 7.0 at 4 °C.

Steady-state Kinetics

The effect of pH on soluble and immobilized urease was determined at different pH values at standard assay conditions with 0.05 M Tris–acetate buffer. The activity assay for soluble and immobilized urease was performed at different temperatures (10°–80 °C) and optimum temperature was determined. The different concentrations of urea were prepared and assayed as described earlier to study the effect of substrate concentration on soluble and immobilized urease. The Michaelis Constant (K_m) values were determined using Lineweaver–Burk plot.

Assay of Blood Urea with Immobilized Urease

The agar tablets were incubated with 1.0 ml of 0.05 M Tris–acetate buffer at 37 °C for 5 min. Two hundred microliter of serum sample was added and was incubated for another 20 min. After the desired time interval, 1.0 ml of this reaction mixture was taken for color development as described for soluble urease and absorbance was recorded spectrophotometrically at 405 nm.

Storage Stability of Immobilized Urease

The soluble and immobilized ureases were stored in 0.025 M Tris–acetate buffer, pH 7.0 and 4 °C for their stability studies. The activity assay was recorded at a regular interval of 1 week. The storage stability was monitored for about 3 months and shelf life was determined.

Results and Discussion

Urease Immobilization

The glass plates, Eppendorf tubes, and tablet strips were used as casts, but the process was too cumbersome with glass plates and Eppendorf tubes. Therefore, tablet strips were preferred for the purpose of casting agar, as it could be easily poured into the tablet strips to obtain the agar tablets of uniform shape and size. The optimum immobilization (51.7%) was obtained at 5% agar (*w*/*v*) and 0.019 mg protein/agar tablet. When agar concentration was raised over 5%, there was not any increase in percent immobilization. At higher concentrations, it was quite difficult to pour agar into the tablet strips. Furthermore, when the concentration was decreased, the proper solidification could not be achieved and agar tablets were very soft and fragile. The time and temperature of solidification was 6 min and 44 °C, respectively. The protein concentration of 0.019 mg per agar tablet yielded good results. The higher concentrations of protein definitely showed some improvement towards percent immobilization; however, this was not economical. The results are shown in Table 1.

Table 1 The standardization of various parameters for optimum immobilization of pigeonpea urease on agar tablets.

Concentration of agar % (*w*/*v*)	Protein (milligram per agar tablet)	Immobilization (%)
2.0	0.015	–[a]
3.0	0.015	25.0
4.0	0.015	38.0
5.0	0.015	47.7
6.0	0.015	42.2
7.0	0.015	38.1
10.0	0.015	–[b]
5.0	0.010	25.2
5.0	0.013	37.0
5.0	0.016	48.0
5.0	0.019	51.7
5.0	0.022	51.9
5.0	0.025	49.1
5.0	0.025	52.0

Percentage immobilization was defined as the (total activity of immobilized enzyme/total activity of the soluble enzyme loaded) ×100.

[a] Agar tablets did not form.

[b] The agar tablets were not uniform in shape and size.

The pigeonpea urease has been immobilized on several matrices like polyacrylamide and sodium alginate: 50% [10]; flannel cloth via polyethyleneimine: 56% [11]; chitosan via glutaraldehyde: 64% [12]; DEAE-cellulose paper strips: 51% [14]; and gelatin: 75% [17]. 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. However, 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.

Effect of pH on Immobilized Urease

The soluble enzyme can have different pH optima from the immobilized enzyme. Depending upon the surface and residual charges on solid matrix and 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 optima of enzyme activity. The pH optima of the soluble and immobilized urease were 7.3 and 7.5, respectively in 0.05 M Tris–acetate buffer (Fig. 1). There was a shift of 0.2 U toward the basic side resulting from the binding of urease. Jack bean urease immobilized on porous glass beads and molecular sieve 4A showed a shift toward the acidic side [23, 24]. However, for jack bean urease, immobilized on a fixed bed reactor showed a shift towards basic side [25].

Effect of Temperature on Immobilized Urease

The soluble urease from pigeonpea has an optimum temperature of 30 °C, whereas that of immobilized urease was 60 °C (Fig. 2). There was a significant increase in optimum incubation temperature when urease was bound to agar, thus, indicating that immobilized urease resist denaturation. It may be mentioned here that under normal conditions of activity in living cells, almost all enzyme exist in a membrane bound form or bound to other macromolecules and are rarely found in the free state as *in vitro* experiments [26]. A similar shift in optimum temperature was observed with chitin bound lactase and jack bean urease on sieve 4A, where

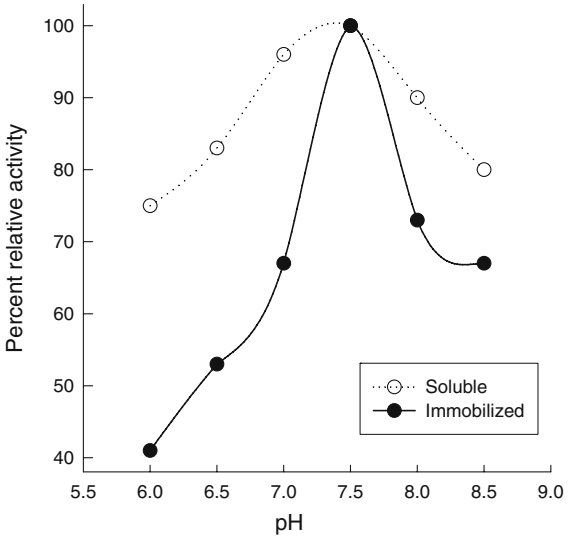

Fig. 1 The effect of pH on the activity of soluble (*empty circle*) and immobilized (*filled circle*) urease. Appropriately diluted aliquot of soluble and immobilized urease were assayed at different pH in 0.05 M Tris–acetate buffers at 37 °C for 10 min. Each point represents the mean of three determinations

even up to 65 °C, the activity was increasing [23, 27]. Enhanced thermal stabilities have been reported for numerous covalently bound enzymes, e.g., jack bean urease on nylon [28].

Effect on Michaelis Constant

The K_m is a measure of affinity of substrate for the active site of an enzyme. Immobilization often results in an increase or decrease in K_m. The K_m for soluble and immobilized urease was determined by LB plot. It was observed that after immobilization, K_m was increased

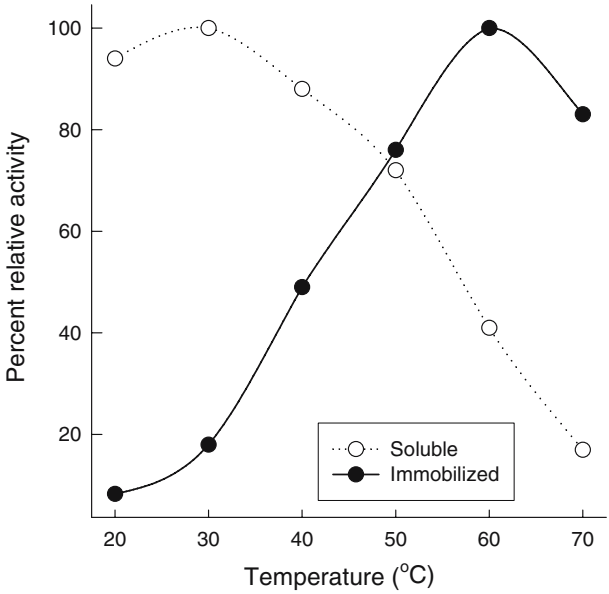

Fig. 2 The effect of temperature on the activity of soluble (*empty circle*) and immobilized (*filled circle*) urease. Appropriately diluted aliquot of soluble and immobilized urease were assayed at different temperatures at pH 7.3 in 0.05 M Tris–acetate buffers for 10 min. Each point represents the mean of three determinations

from 3.23 to 5.07 mM (results not shown). The apparent increase in K_m might be due to changes in the accessibility of substrate to the active site as there are diffusional resistances due to agar gel. The significant changes (increase) in K_m upon immobilization have been observed most commonly in case of encapsulation and adsorption methods [12, 14, 29, 30].

Assay of Blood Urea with Immobilized Urease

The agar tablets were subsequently used to assay blood urea of some patients from local clinical pathology laboratory. The elevated levels of urea are clinically significant in many renal disorders such as uremia, kidney malfunctioning, etc. Urease immobilized on agar tablets offers an easy and inexpensive method to assay urea in various pathological conditions. The values for the urea concentration in the clinical blood samples determined with immobilized urease compare favorably with those obtained by the clinical pathology laboratory. The results are shown in Table 2.

Storage Stability of Beads

The $t_{½}$ of immobilized urease was approximately 53 days when kept in 0.025 M Tris–acetate buffer, pH 7.0 at 4 °C and that for the soluble urease was 21 days under identical condition (data not shown). Immobilization of urease by various methods has led to stability in earlier studies [10–12, 14, 29]. The agar tablets showed linearity with respect to the activity indicating the homogenous distribution of the enzyme in the polymer.

Conclusion

Immobilization using agar as matrix offers several advantages over other materials: easy preparation procedure, low cost, and good mechanical stability. Also importantly, the diffusion coefficient of substrates in agar is much higher than Ca–alginate, K–carrageenan, and gelatin [32]. Therefore, agar matrix offers the least resistance to mass transfer, which would have profound applications in Bioreactors.

Table 2 Estimation of urea in serum using pigeonpea urease immobilized on agar tablets and a comparison with autoanalyzer®.

Serum samples	Immobilized urease (mg/dl)	Autoanalyzer® (mg/dl)
1	33.0±0.9	35.0
2	37.1±0.8	40.0
3	38.0±1.1	40.0
4	35.2±1.0	37.3
5	23.5±0.3	25.6
6	38.1±0.8	40.9
7	32.0±0.7	33.3

The values were determined with immobilized urease on agar tablets. Data were the mean values and standard deviations of three independent experiments.

Acknowledgements Facilities provided by School of Biotechnology are gratefully acknowledged. S. K. and R.C.R.K. are thankful to Council for Scientific and Industrial Research and Indian Council of Medical Research, New Delhi, India for financial support in the form of Senior Research Fellowships.

References

1. Katchalski-Katzir, E. (1993). *Trends in Biotechnology, 11*, 471–478.
2. Hogan, M. E., Swift, I. E., & Done, J. (1983). *Phytochemistry, 22*, 663–667.
3. Bailey, C. J., & Boulter, D. (1970). *European Journal of Biochemistry, 17*, 460–466.
4. Polacco, J. C. (1977). *Plant Science Letters, 10*, 249–255.
5. Mobley, H. L., & Hausinger, R. P. (1989). *Microbiological Reviews, 53*, 85–108.
6. Dixon, N. E., Gazzola, C., Blakeley, R. L., & Zerner, B. (1975). *Journal of the American Chemical Society, 97*, 4131–4133.
7. Holst, O., & Mattiasson, B. (1991). *Biosensors & Bioelectronics, 6*, 101–108.
8. Lee, K. B., Boadi, D. K., & Neufeld, R. J. (1995). *Journal of Theoretical Biology, 175*, 295–303.
9. Das, N., Prabhakar, P., Kayastha, A. M., & Srivastava, R. C. (1997). *Biotechnology and Bioengineering, 54*, 619–623.
10. Das, N., Kayastha, A. M., & Malhotra, O. P. (1998). *Biotechnology and Applied Biochemistry, 27*, 25–29.
11. Das, N., & Kayastha, A. M. (1998). *World Journal of Microbiology & Biotechnology, 14*, 927–929.
12. Kayastha, A. M., & Srivastava, P. K. (2001). *Applied Biochemistry and Biotechnology, 96*, 41–53.
13. Kayastha, A. M., Srivastava, P. K., Miksa, B., & Slomkowski, S. (2003). *Journal of Bioactive and Compatible Polymers, 18*, 113–124.
14. Reddy, K. R. C., Srivastava, P. K., Dey, P. M., & Kayastha, A. M. (2004). *Biotechnology and Applied Biochemistry, 39*, 323–327.
15. Das, N., Kayastha, A. M., & Srivastava, P. K. (2002). *Phytochemistry, 61(5)*, 513–521.
16. Srivastava, P. K., & Kayastha, A. M. (2000). *Plant Science, 159*, 149–158.
17. Srivastava, P. K., & Kayastha, A. M. (2001). *Journal of Molecular Catalysis. B, Enzymatic, 16*, 81–89.
18. Clark, L. C. Jr. (1962). *Annals of the New York Academy of Sciences, 102*, 29–45.
19. Kircka, L. J., & Thorpe, G. H. G. (1986). *Trends in Biotechnology, 4*, 253–258.
20. Klein, M. D., & Langer, R. (1986). *Trends in Biotechnology, 4*, 179–186.
21. Watanabe, S., Shimizu, Y., Teramatsu, T., Murachi, T., & Hino, T. (1988). *Methods in Enzymology, 137*, 545–551.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *The Journal of Biological Chemistry, 193*, 265–280.
23. Iyengar, L., Bajpai, P., & Rao, A. V. S. P. (1982). *Indian Journal of Biochemistry & Biophysics, 19*, 130–134.
24. Weetall, H. H., & Hersh, L. S. (1969). *Biochimica et Biophysica Acta, 185*, 464–465.
25. Maynihan, H. J., Lee, C. K., Clarck, W., & Wang, N. H. L. (1989). *Biotechnology and Bioengineering, 34*, 951–963.
26. Mosbach, K. (1971). *Scientific American, 224*, 26–33.
27. Stanley, W. L., Watters, G. G., Keiley, G., Chan, B. G., Goribaldi, J. A., & Schade, J. E. (1976). *Biotechnology and Bioengineering, 18*, 439–445.
28. Sundaram, P. V., & Hornby, W. E. (1970). *FEBS Letters, 10*, 325–329.
29. Srivastava, P. K., Kayastha, A. M., & Srinivasan (2001). *Biotechnology and Applied Biochemistry, 34*, 55–62.
30. Krajewska, B., Leszko, M., & Zaborska, W. (1990). *Die Angewandte Makromolekulare Chemie, 179*, 21–33.
31. Reddy, K. R. C., & Kayastha, A. M. (2006). *Journal of Molecular Catalysis. B, Enzymatic, 38*, 104–112.
32. Nakarani, M., & Kayastha, A. M. (2007). *Oriental Pharmacy and Experimental Medicine, 7*, 79–84.