Immobilization of urease in gelatin beads for urea estimation

Mohammed A.J.Al-Khafaji
Babylon University, College of Scince, Biology Dept.
Hilla-Iraq
Mufeed J.Ewadh
Babylon University, College of Medicine,Biochemistry Dept.
E-mail: mewadh@yahoo.com

(NJC)

(Received on 20/5/2008) (Accepted for publication 26/10/2008)

Abstract

Urease was immobilized in gelatin beads. The optimum immobilization (79% activity) was observed at 4 C˚ with protein concentration of 1.0 mg/ml. the apparent optimum pH shifted from 7.5 to 6.5. Immobilized urease showed optimum activity at 60 C˚ compared with 40 C˚ for the soluble urease.The immobilized urease remained very active over long period of time and this enzyme lost about 40% of its original activity over the period of 60 days for storage at 4C compared with soluble urease which lost about 75% its activity in the same conditions. Blood urea estimation is carried out with immobilized enzyme beads compared with soluble enzyme and the beads can be used repeatedly for this purpose making it economical procedure compared to standard chemical method.

الخلاصة

تم تقيد أنزيم اليوريز باستخدام الجيلاتين وكانت نسبة التقيد 79% اظهر الأنزيم المقيد أعلى فعاليـة عند رقم هيدروجيني 6.5 مقارنة بالأنزيم الحر7.5. واظهر أعلى فعالية عند درجة حـرارة 60 ˚م مقارنـة بالأنزيم الحر (40 ˚م).بقي الأنزيم المقيد فعال لفتره طويله حيث فقد حوالي 40% من فعاليته عند خزنه لمدة 60 يوم مقارنة بالأنزيم الحر الذي فقد 75% من فعاليته عند خزنه بنفس الظروف. تم تقدير نـسبة اليوريـا بالدم باستخدام الأنزيم المقيد وكانت الطريقة كفوءة واقتصادية.

Introduction

Urease (E.C 3.5.1.5),a nickel-dependent metalloenzyme, catalyses the hydrolysis of urea to form ammonia and carbon dioxide. Hydrolysis of one molecule of urea results in the release of two molecules of ammonia and one molecule of carbon dioxide.

$$(NH_2)_2CO + 3H_2O \xrightarrow{\text{Urease}} CO_2 + 2NH_4OH$$

The enzyme plays an important role in the determination of urea in blood, urine and in wastewater, in process of dialysis for removal of urea from blood in the treatment of uremia, etc [(1)].

Approximately half a million patients worldwide are being supported by haemodialysis[(2)].

The conventional artificial kidney is bulky, heavy, complex and expensive and difficult to handle, limiting the motility of the patients.

The use of microencapsulated urease is being developed as useful system in kidney machines to maintain the urea level in the blood patients suffering alterations in kidney function and in an attempt to construct a portable/ wearable artificial kidney [3].

The judicious and efficient utilization of enzyme in industry requires that they be physically or chemically immobilized. Enzyme immobilization has been a subject of attention for many years [4,5].

The use of enzymes is often limited due to their high cost, availability in small amounts, instability and limited possibility of economic recovery of these soluble biocatalysts from a reaction mixture. Recent development in the field of biotechnology for immobilizing enzymes can overcome some of these problems.

Immobilization of urease from different sources; Jack bean, *C.vulgaris*, Pigeonpea colocynth has been studied intensively [6,7,8,9] some of the support material used for immobilization are gelatin [8,9], DEAE cellulose paper [10], or physically entrapped/ encapsulated solids, such as cross-linked gel like, polyacrylamide and calcium alginate [11], chitosan [12], and coupling alkylamine and arylamine glass [13].

Gelatin, which is protein polymer, has been previously used to immobilize enzymes [14]. Enzyme immobilization on gelatin can mainly be achieved by means of the cross-linking reactions between the free amino group of gelatin and the enzyme molecule, through crosslinkers, to form a covalent linkage. The cost of this support material compared with the cost of other materials, such as polyacreylamide and algenic acid, is low.

The aim of this study was to immobilize urease on gelatin beads to investigate some factors in comparison with the free enzyme which may be used to gain knowledge on the effects of immobilization on enzyme properties. The immobilized urease has been used for the assay of blood urea in clinical samples.

Materials & Methods

Glutaraldehyde, gelatin powder was supplied from BDH chemical company, urea was supplied from Fisher scientific company. All other chemicals and solvents were supplied BDH chemical company. Urease enzyme from bioMerieux.

Preparation of gelatin gel

Gelatin (0.6 g) was dissolved in 10 ml of 50 mM phosphate buffer (pH 7.5) by heating at 50˚ C with continuous stirring for 5 min. to obtain clear solution. This solution was cooled and the solidified mixture was stored at 4 C˚ prior to immobilization the mixture was heated to 60 C˚ and then slowly brought to 27 C˚ in order to obtain a clear solution.

Immobilization of urease

A clear solution of gelatin (60 mg/ml), 0.1mg/ml enzyme and 0.6% (v/v) glutaraldehyde was mixed together and stirred constantly at 27 C˚ and poured in a (7 cm×4 cm) glass plate to prepare a thin film of the enzyme(19). The film was stored at 4 C˚ for 18 h for complete cross-linking. The immobilized enzyme film was washed thoroughly with 50 mM phosphate buffer (pH 7.5) to remove any unbound enzyme and then cut into small blocks before subsequent experiments.

Soluble urease assay

Activity was assayed in 50 mM phosphate buffer (pH 7.5). an aliquot (0.8 ml) of buffer and 0.2 ml of 500 mM urea in the same buffer were brought to 37 C˚. the reaction was started by adding 0.2 ml of suitably diluted enzyme. Following incubation for 10 min. the reaction was terminated by the addition of 10% (w/v)

trichloroacetic acid. The total reaction mixture was transferred to a measuring flask (50 ml) and the volume was made to 50 ml with distilled water after adding 1.0 ml of Nessler's reagent as described earlier [15]. The amount of ammonia liberated was measured at 405 nm in spectronic 21 spectrophotometer. An enzyme unit is defined as the amount of enzyme required to liberate 1 µmol of ammonia/min under test condition defined above (37C˚, 50mM phosphate buffer pH 7.5, 500 mM urea).

Immobilized urease assay

For assay of immobilized enzyme the beads were incubated at 37 C˚ for 15 min. in standard assay medium comprising of 50 mM phosphate buffer (pH 7.5) containing 500 mM urea. Following incubation, an aliquot of 1.0 ml was withdraw from the reaction mixture and assayed as described above. The beads were recovered from the reaction mixture, washed thoroughly with buffer and stores at 4 C˚. The percentage of immobilized is defined as the (total activity in immobilized bead/total activity of the soluble enzyme loaded) ×100.

Protein Estimation

Protein was estimated by the method of Bradford [16].

Stability Test

For storage stability studies, soluble and immobilized alginate beads urease were kept in phosphate buffer PH 7 at 4 C˚. The activity of immobilized and soluble urease determined on different days by the method described above.

Physical characterization of enzyme

Effect of pH on the activity of soluble and immobilized urease was studied in the different buffer solution of pH ranging from 4 to 10 (pH 4-6, 50 mM acetate buffer, pH 6.5-8, 50 mM phosphate buffer,pH 8.5-10 tris-HCl buffer).

The effect of temperature on the activity of both enzyme was studied between 20-60 C˚ at pH 7, 50 mM phosphate buffer.

Assay of blood urea with immobilized urease

Beads(2-4) were incubated with 1.0 ml of 50mM phosphate buffer(PH 7.5) at 37C for 5 min.The reaction was started by the addition of 0.2 ml of serum.After 20 min 1.0 ml of this reaction mixture was withdrawn for color development as described for soluble urease and absorbance was recorded spectrophotometrically at 405 nm.

Results & Discussion

Urease immobilization

The general behavior of urease immobilized on gelatin beads under different conditions of immobilization was studied. The percentages of immobilization achieved with various gelatin concentration, glutaraldehyde concentration and protein concentration are summarized in table 1.

Its clear from the data that the optimum immobilization 79% is obtained at 60 mg/ml gelatin 0.1 mg of enzyme 0.6 (w/v) glutaraldehyde.

Table (1): Optimum conditions for the immobilization of urease on gelatin at 4 C˚.

Gelatin (mg/ml)	Enzyme (mg/ml)	Glutaraldehyde	immobilization %
40	0.1	0.6	52.90
50	0.1	0.6	62.60
60	0.1	0.6	79.04
70	0.1	0.6	69.50
60	0.3	0.6	50.20
60	0.2	0.6	47.30
60	0.1	1	68.79
60	0.1	2	38.30
60	0.1	3	27.20

Effect of pH on immobilized urease

The effect of pH on the activity of free and immobilized urease is shown in Figure-1. The pH optima of the soluble and gelatin-bound urease were 7.5 and 6.5 respectively in 50 mM phosphate buffer. There was a shift of 1 units towards an acidic pH value resulting from the binding of urease. A similar shift in pH optimum has been reported for *Citrullus colocynthis* urease immobilized on gelatin beads [9].

However, Jack bean urease immobilized on porous glass beads the pH optimum of 7.2 for the soluble enzyme compared with 6.1 for immobilized enzyme[17] in the case of alkylamine immobilization of Pigeonpea urease a shift was observed from 7.3 (soluble) to 6.5 (immobilized) [13].

However, immobilized enzyme maintained a higher relative activity than free urease at both lower and higher pH levels, indicating that the immobilized enzyme was less sensitive to pH changes than the free urease.

The behaviour of an enzyme molecule may be modified by its immediate micro-environment. An enzyme can have an altered pH optimum upon immobilization on a solid matrix in relation to its pH optimum in solution. Depending on the surface and residual changes 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 [12, 13].

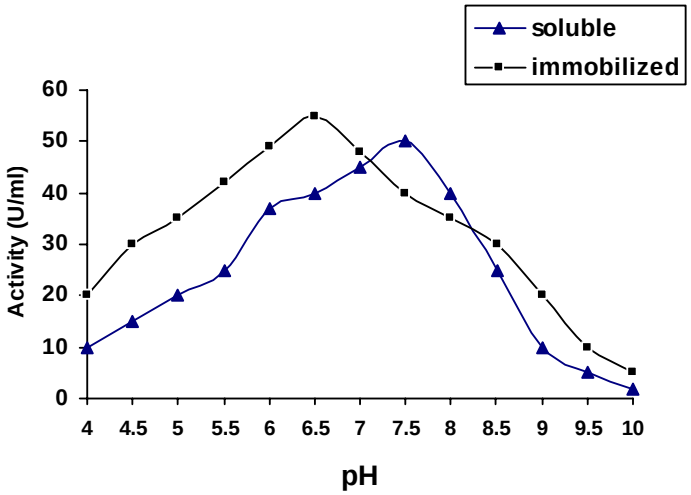

Figure (1): Effect of pH on soluble and gelatin immobilized urease.

Optimum temperature

Results of the effect of temperature on gelatin-bound urease and soluble urease are shown in Figure-2. free urease has an optimum temperature of 40 C˚. Whereas gelatin-bound urease was 60 C˚. The increase in temperature optima for activity of immobilized enzyme could be due to the fact that actual temperature in the micro-environment of the gel matrix was lower than in the bulk solution [18]. It could be due to the fact that under normal conditions in living cells, most of the enzyme are either bound to membrane or to other macromolecules and rarely exists in free form as invitro experiments [18]. The similar increased optimum temperature of immobilized *urease* was also observed by others [19, 20].

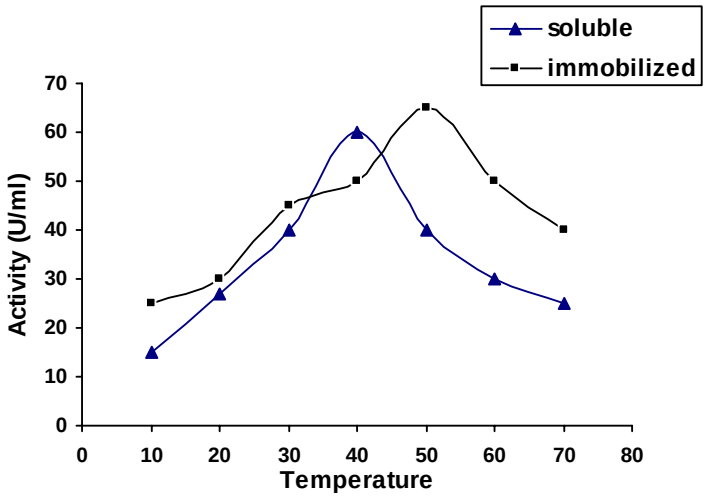

Figure (2): Effect of temperature on the activity of both soluble and gelatin immobilized urease.

Storage stability

The loss of activity, for soluble urease after 60 days of storage at 4 C˚ was 75% and in comparison to gelatin-urease the lose was 40% (Fig.3). This clearly signifies that coupling of enzyme to gelatin matrix had tremendously improved the storage stability of urease and this is one of the biggest advantages of urease immobilization on gelatin.

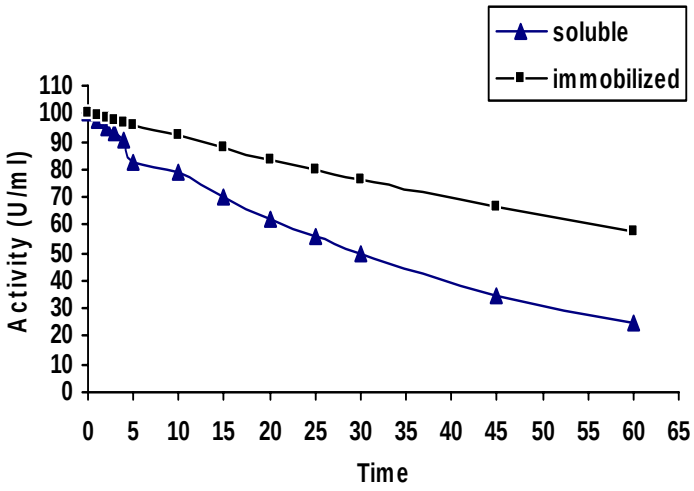

Figure (3): Storage stability studies of gelatin-immobilized urease.

Assay of blood urea with immobilized urease

The gelatin beads were subsequently used to assay blood urea of some patients from Al-Mahaweel hospital. There are several potential uses for immobilize urease e.g the estimation of urea content in biological fluid is generally performed using either chemical or biological methods that use soluble urease kit. Each method is expensive and non reusable. Immobilized urease overcomes some of these problems. The results are shown in (Table-2).

Table (2): Estimation of blood urea (mg/dL) of some clinical samples with gelatin-immobilized urease compared with urea estimated by chemical method.

Serum Sample	Immobilized urease	Chemical method
1	38.00	40.00
2	97.00	97.12
3	85.19	85.60
4	81.80	80.00
5	33.00	35.00
6	22.00	24.00
7	100.00	98.00
8	218.00	215.00
9	225.00	221.00
10	100.00	98.00

References

1. Ayhan, F.; Ayhan, H.; Piskin, E. & Tanyolac, A., ***Bioresearch Technology***, 2002, **81**, 131.
2. Marzadori, C.; Milettis, S.; Gessa, C. & Civrilli, S., ***Soil Biol. Biochem.***, 1998, **30**, 1485.
3. Kayastha, A.M. J. & Das, N., ***Biochemical Education***, 1999, **27**, 114.
4. Karajewska, B.; Leszko, M. & Zaborska, W., ***Post. Fiz. Med.***, 1988, **23**, 115.
5. Laska, J.; Wlodarczyk, J. & Zaborska, W., ***Journal of Molecular Catalysis B: Enzymatic***, 1999, **5**, 549.
6. Mosbach,M., ***Sci.Am.***, 1971, **16**, 129.
7. Fahmy, A.S.; Bagos, V.B. & Mohammed, T.M., ***Bioresearch Technology***, 1998, **64**, 121.
8. Das, N.; Kayastha, A.M.; Malhotra, O.P., ***Biotechnology Applied Biochemistry***, 1998, **27**, 25.
9. Al-Khafaji, M.A.J. (2007). Ph. D. Thesis. Baghdad university.
10. Reddy,K.R.C.; Srivastava,P.K.; Dey, P. M.; Kayastha, A. M., ***Biotechnol Appl.Biochem.***, 2004, **39**, 323.
11. Das, N.; Kayastha, A.M.; Malhotra, O.P., ***Applied Biochem.***, 1998, **1**, 25
12. Kayastha, A.M. & Srivastava, P.K., ***Appl. Biochem. Biotechnol***., 2001, **96**, 41.
13. Reddy, K.R.; Kayastha, A.M., ***Journal of Molecular Catalysis B: Enzymatic***, 2006, 104.
14. Srivastava, P.K.; Kayastha, A.M. & Srinivasan, K., ***Biotechnol. Biotechnol. Appl. Biochem.,*** 2001, **34**, 55.
15. Prakash, O. & Upadhyay, B., ***Plant Sci.***, 2003, **164**, 189.
16. Bradford, M.M., ***Anal. Biochem***., 1976, **72**, 284.
17. Lyengar, L.; Baj pai, P. & Prabhakara, R.A.V., ***Indian J. Biochem. Biophys***., 1982, **19**, 130.
18. Prakash, O.; Puliga, S. & Sheo, L.; Upadhyay, B., ***Biotechnology & Bioprocess Engineering***, 2007, **12**, 131.
19. El-Shora, H.M., ***Bot. Bull. Acad. Sin***., 2001, **42**, 251.
20. Mulagalapalli, S.; Kumar, S.; Kalathur, R.C.R.; Kayastha, A.M., ***Appl. Biochem. Biotechnol***., 2007, **142**, 291.