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Development of a Urea Potentiometric Biosensor Based on Gelatin-Immobilized Urease

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ABSTRACT

Urease (typeIII) purified from Jack bean was immobilized on gelatin beads via cross-linking with glutaraldehyde. The maximum immobilization (70.82%) was observed with a half-life of 385 days and there was practical stability for over a period of 50 days. These beads could be reused more than 10 times (with 24 h intervals) without much loss of enzyme activity (i.e. less than 12%). The immobilized beads were used for the preparation of a new urea biosensor developed from the potentiometric pH glass electrode and the reference calomel electrode with major advantages such as long term operational and storage stability, response time and linear range. The beads, as well as the biosensor, were used to analyse the urea content in clinical samples from the local pathology laboratory. The results obtained with the biosensor were similar to those obtained with the various commonly employed biochemical/ autoanalyzer® methods. These immobilization studies also have a potential role in haemodialysis machines that are used to maintain the urea level in kidney patients and in the construction of a portable or wearable kidney.

Key words: urease, urea, immobilization, gelatin, potentiometric biosensor

INTRODUCTION

Urea is a major metabolic product of protein, and its determination is important for both measurements of glomerular filtration rate and renal function testing (Wei and Shih, 2001). The first product of urea hydrolysis by urease is ammonium carbamate ($H_2NCO_2NH_4$) rather than ammonium carbonate (Huang and Chen, 1992). Ammonium carbamate, however, rapidly breaks down into ammonium carbonate and then to ammonia and carbon dioxide in the final step. Urease (urea aminohydrolase, E.C.3.5.1.5), a nickel-dependent metalloenzyme, was the first enzyme to be obtained in crystalline form. The properties of ureases from plants and microorganisms have been reviewed extensively (Sansubrino and Mascini, 1994; Kayastha and Das, 1999; Wei and Shih, 2001; Jedner *et al.*, 2002). So far the crystal structures of ureases from *Klebsiella aerogenes* have been determined (Jedner *et al.*, 2002). A coordination of the nickel ion by five or six donor atoms is observed.

Both nickel ions in urease are involved in the catalytic reaction. It was suggested in two different schemes; (i) a coordination of the carbonyl oxygen of urea to Ni (1) followed by a nucleophilic attack of a hydroxide ion coordinated to Ni (2). In contrast, (ii) urea bridges the two metal ions via its carbonyl oxygen and an amide

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group. The nucleophilic attack occurs via a bridging hydroxide ion.

Urease has been used in an immobilized form in kidney machines for blood detoxification. According to one report approximately half a million patients worldwide are being supported by haemodialysis (Srivastava *et al.*, 2001). The conventional artificial kidney is bulky, heavy, complex and expensive, and difficult to handle, limiting the mobility of the patients. The use of microencapsulated urease is being developed as a useful system in kidney machines to maintain the urea level in the blood of patients suffering disruption in kidney function and in an attempt to construct a portable/wearable artificial kidney. The immobilization of enzymes to synthetic polymer membranes is also of particular interest because of the possibilities for their application in biosensors and membrane bioreactors. Several biosensors with immobilized ureases have been described for blood urea assay. These include a recent method of entrapment of urease inside reversed micelles as a method of immobilization and using a glass electrode as a sensor (Magalhães and Machado, 1998; Srivastava *et al.*, 2001).

Potentiometric biosensors, based on the detection of either ammonium ion, ammonia gas, carbon dioxide or pH change produced by the enzymatic reaction, are among the most attractive biosensors for urea, because of the simplicity of their construction procedure and the general availability of the instrumentation required for their utilization. Enzyme electrodes that measure the produced ammonium ions have been developed. Immobilization of relevant enzymes at the surface of the sensing electrode is one of the crucial steps in the construction of a biosensor. A new urea sensor has been described, which makes use of entrapment of urease inside reverse micelles as a method of immobilization of the enzyme and uses a glass electrode for the purpose of sensing. The aim of the work was to immobilize urease on gelatin beads and to compare the kinetic properties of native soluble urease with the immobilized enzyme. Both types of urease were used for the assay of blood urea in clinical samples. Furthermore, the immobilization on gelatin was used to design a new urea biosensor for estimation of urea in biological samples.

MATERIALS AND METHODS

Enzyme and chemicals.

Urease(EC.3.5.1.5, typeIII, 20,000 units) from jack beans,Tris(hydroxymethyl) aminomethane acetate salt, potassium iodide and mercury(II) iodide were purchased from Sigma Chemical Co.(USA.). Urea, glutaraldehyde, sodium carbonate, sodium hydroxide, copper sulfate pentahydrate, potassium sodium tartate and gelatin were obtained from Ajax Finechem Co.(USA.). Trichloroacetic acid was purchased from Riedel-deHaen Co. Folin-Ciocalten's phenol reagent and bovine serum albumin (BSA) were obtained from Fluka Chemical Co.(Switzerland) All reagents were of analytical reagent grade used without further purification and were prepared in double distilled water from an all-glass assembly. Pooled serum samples were obtained from a local pathology (Somdejprapinklao Hospital, Pathology Laboratory, Bangkok, Thailand).

Urease assay and the calibration of Nessler's reagent.

Nessler's reagent was prepared and the calibration curve was constructed as described previously (Panpae *et al*., 2005). The yellow colour produced is measured at 405 nm. A blank is run without enzyme and suitable correction is applied. An enzyme unit is defined as the amount of enzyme required to liberate 1 μmol ammonia in 1 min. under the test conditions (0.1 M urea, 0.05 M Tris acetate buffer, pH 6.5 and 7.3, at 37°C).

Preparation of gelatin gel and immobilization of urease.

Gelatin (0.1 g) was dissolved in 20 ml of 50 M Tris acetate buffer (pH 6.5 and 7.3) by heating at 60°C with continuous stirring for 1 h to obtain clear solutions. These solutions (pH 6.5 and 7.3) were cooled and the solidified mixtures were heated to 60°C and then slowly brought to 27°C in order to obtain clear solutions. At each pH, a clear solution of gelatin (30 mg/mL), 0.414 mg/mL enzyme and 1% (v/v) glutaraldehyde were mixed together and stirred for 10 sec. Immediately, the mixture was cast in hollow plastic cylinders (0.6 cm × 0.6 cm) and kept at 4°C for 24 h. The gelatin beads were then washed with 50 mM Tris acetate buffer (at each pH) to remove any unbound enzyme. The beads were stored at 4°C in 50 mM Tris acetate buffer (pH 6.5 and 7.3).

Enzyme activity assay.

For both soluble and immobilized urease activity, the amount of ammonia liberated during a fixed time period at a saturating concentration of urea was determined, as described previously (Panpae *et al*., 2005; Kayastha and Das, 1999). To start the reaction 0.1 M urea was added to the enzyme, which was suitably diluted in 50 mM Tris acetate buffer (pH 6.5 and 7.3). Following incubation for 10 min the reaction was terminated by the addition of 10% (w/v) trichloroacetic acid. Colour was developed in the supernatant using Nessler's reagent. An enzyme unit was calculated from the amount of enzyme required to liberate 1 mmol of ammonia/min under the test conditions (0.05 M Tris acetate buffer (pH 6.5 and 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 containing 0.1 M urea at 37°C with intermittent shaking. Following incubation for the desired time, an aliquot of 1 mL was withdrawn from the reaction mixture and assayed as described above (Kayastha and Das, 1999).

Blood urea determination with immobilized and soluble urease.

2 beads (containing *ca* 1 unit of activity) were preincubated in 1.90 mL of assay buffer (0.05 M Tris acetate (pH 7.3)) for 5 min at 37°C. The reaction was started by the addition of 0.1 mL of serum. After 20 min, 1.0 mL of 10% (w/v) trichloroacetic acid was added. The precipitated proteins were removed by centrifugation (7000 rev min^{-1}) for 20 min and the colour was developed with the supernatant (0.1 ml), as for the soluble enzyme. For the assay of blood urea with soluble urease, the procedure was performed as described above by using 1.0 mL of urease solution (*ca* 1 unit) instead of 2 beads of gelatin-urease.

Construction of a urea biosensor.

Potentiometric measurements were made with a Mettler Toledo MPC 227 model equipped with a Radiometter PHG 201-8 glass electrode coupled to a calomel electrode. All the measurements were taken at constant temperature while using a Fisher scientific unstirred digital bath Isotemp 228 model.

Evaluation of the biosensor and assay of urea in serum.

20 immobilized beads were kept in a dialysis tube (2.5 cm × 1.6 cm). The bottom of the tubing was tied with a thread. The glass pH electrode was inserted into the open end of the tubing and this end of the dialysis tubing was then tied above the electrode bulb. Buffer (3.0 mL of 0.05 M Tris acetate (pH 7.3)) placed in a beaker was stirred at a moderate speed. The electrode was immersed in the solution and when the electrode potential across the two leads of the composite glass electrode reached a stable value and then the urea solution of known concentration was added. The stable value reached was recorded. Various known concentrations (20, 40, 60, 80 and 100 mg/dL) of urea were used so that a calibration curve could be plotted. To measure the urea content of blood serum, 10.0 mL of 0.05 M Tris acetate buffer

(pH 7.3) was placed in a beaker. Serum (100 μL) was added and the electrode potential was measured as described above. Urea concentration was subsequently determined using the calibration plot. All analyses were done in triplicate.

Storage stability of the immobilized preparation.

For storage stability studies, immobilized gelatin beads were kept at 4°C. The activity of the immobilized urease was determined on different days by the method described above. For each assay, fresh beads were used. The haft-life of immobilized urease was calculated from a plot of log% residual activity vs. time: $\log A_t = \log A_0 - \lambda / 2.303\ t$ and $t_{1/2} = \ln 2 / \lambda$ where A_t and A_0 are enzyme activities at any time and initial time, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the linearity ($r^2 = 1.000$) of the calibration of Nessler's reagent with a standard solution of ammonium chloride.

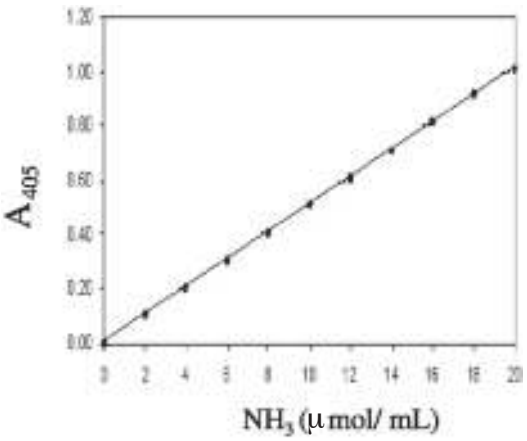

Figure 1 Calibration curve of Nessler's reagent with standard ammonium chloride.

Effect of the selected pH on immobilized urease.

The effect of the selected optimum pH on the activity of the free and immobilized urease is shown in Table 1. The pH optimum of the gelatin-bound urease was 7.3 (70.82% immobilization) in 0.05 M Tris acetate buffer. In the case of chitosan immobilization of pigeonpea urease (Magalhães and Machado, 1998), a shift was observed from 7.3 to pH 8.5 in 0.05 M Tris acetate buffer. The behavior of an enzyme molecule may be modified by its immediate micro-environment (Srivastava *et al.*, 2001). An enzyme can have an altered pH optimum upon immobilization on a solid matrix in relation to its pH optimum in solution.

Storage stability of beads.

The stability of urease was enhanced quite significantly upon immobilization. The haft-life of immobilized urease was approximately 385 days when kept in 0.05 M Tris acetate buffer (pH 7.3) at 4°C compared with a haft-life of 24 days for the soluble urease under identical conditions (Table 2 and Figure 2). Furthermore, the storage stability of gelatin-immobilized urease is better compared with urease immobilization using other materials, i.e. 70, 75 and 110 days for cotton cloth, calcium alginate beads and chitosan beads, respectively (Srivastava *et al.*, 2001; Panpae *et al.*, 2005). The beads showed good linearity with respect to the activity. The indicated the homogeneous distribution of the repeated use after 50 days for blood urea estimation, which still

Table 1 Effect of the selected pH on gelatin-immobilized urease.

pH	Absorbance	NH_3 (μmol/mL)	Activity before immobilization	Activity after immobilization	% immobilization
6.5	0.7000	69.03	165.60	96.57	58.32
7.3	0.4900	48.32	165.60	117.28	70.82

retained 90.70% residual activity compared with 21.37% residual activity for soluble urease. This gelatin-immobilized enzyme could be used well for further estimations of blood urea assay (Table 3).

Analysis of blood serum.

The calibration of protein assay with BSA standard is shown in Figure 3. The gelatin beads immobilized with urease were subsequently used to assay the blood urea samples of some

Table 2 Comparison of gelatin-immobilized and soluble urease on the percentage of residual activity.

Time (days)	% Residual activity	
	Immobilized urease	Soluble urease
0	100.00	100.00
1	100.00	93.36
2	99.75	91.06
3	99.50	89.09
4	99.25	87.11
5	98.75	80.21
10	98.25	72.65
15	97.65	61.50
20	96.73	52.60
25	95.73	44.71
30	94.73	38.79
45	91.96	25.97
50	90.97	21.37
$t_{1/2}$	385	24

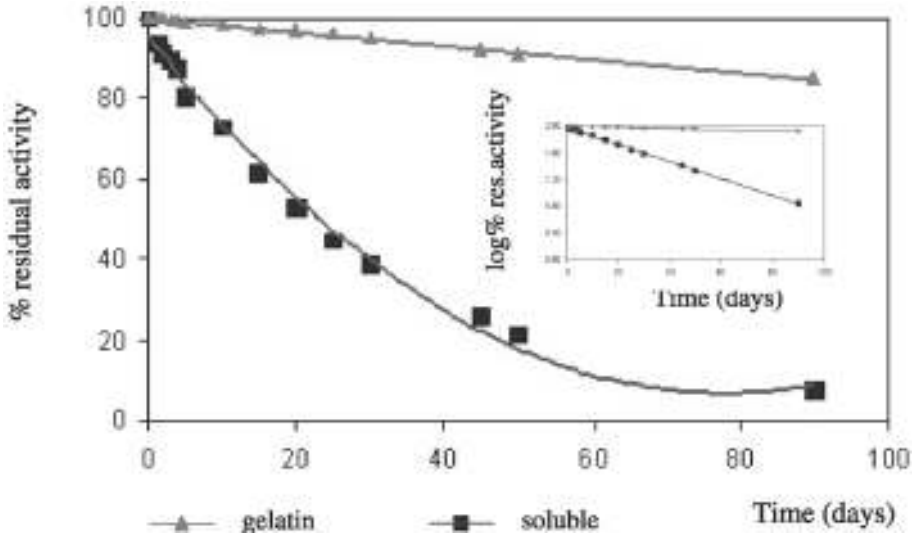

Figure 2 Storage stability of gelatin-immobilized jack bean urease. Beads were stored in 0.05 M Tris acetate buffer (pH 7.3) at 4°C. The inset shows the semi-log plot of the data (res.: residual).

patients from the Somdejprapinklao General Hospital, Pathology Laboratory. Conditions (such as number of beads and incubation period) were worked out using 100 mg of urea/dl, which is much higher than normal physiological range (20-40 mg/dl). A calibration curve with urea concentration ranging from 0-100 mg/dl is shown in Figure 3. The urea assay of serum samples is given in Table 4.

Assay of urea in serum using biosensor.

The biosensor prepared from gelatin beads was used sucessfully for estimation of serum urea samples. The calibration curve obtained by plotting the potential difference (mV) across the two leads of the separated glass electrode against the concentration of urea is shown in Figure 4 and the response time with the present experiments (0.01 M urea at 7.3) is shown in Figure 5. The response time observed in the present study was comparable with those reported for other urea biosensors (Magalhães and Machado, 1998; Srivastava *et al.*, 2001; Schitogullari and Uslan, 2002). Several parameters were evaluated to ascertain the optimum performance of the base line, slope of the calibration curve and reliability of the biosensor. The detection limit in this case was approximately 5 mg/dl. At different time intervals, variation in electrode potential was monitored using 0.01 M urea solution. A little loss of activity was observed over a period of 4-7 weeks and the reproducibility of the urea assay for the serum samples obtained from our biosensor was very good compared with a standard autoanalyzer®

Table 3 Assay of serum urea content using reusable gelatin beads.

Time (days)	Urea[a](mg/dL)				Automatic analyzer®
	Absorbance at 405 nm		Biosensor		
	Gelatin beads	soluble urease	Gelatin beads	soluble urease	
0	36.19	35.95	36.45	36.17	
25	36.22	36.78	36.58	37.23	36.13
50	36.80	46.23	37.13	45.89	

[a] Values obtained are presented as the means from three repeats.

Table 4 Determination of serum urea content using soluble urease, gelatin beads, the biosensor and an autoanalyzer®.

Sample	Urea[a] (mg/dl)			
	Soluble urease	Gelatin beads A_{405}	Biosensor	Automatic analyzer®
1	22.92	22.50	21.29	21.38
2	38.33	37.50	35.63	35.76
3	34.17	33.75	32.31	32.14
4	55.42	54.17	53.72	54.06
5	38.75	38.33	38.88	38.73
6	28.33	29.58	29.12	28.31
7	39.58	38.75	37.38	37.45
8	35.83	37.08	37.71	36.13
9	23.33	22.50	21.44	22.18
10	56.25	56.25	55.35	55.04

[a] Average of three determinations.

[b] The normal physiological range: 20-40 mg/dL

(Table 3). The measurements (average of three determinations of each serum sample) were carried out to evaluate the urea concentration determined with the potentiometric biosensor versus the value provided by the clinical analysis laboratory (autoanalyzer®). For the two methods, urea biosensor and spectrophotometric method, the relative standard deviation were 3.2 and 3.7%, respectively.

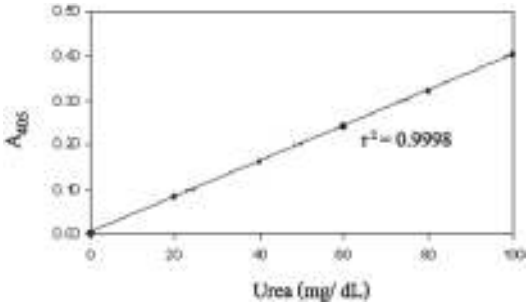

Figure 3 Calibration curve for urea assay (mg/ dL) using immobilized urease in gelatin beads.

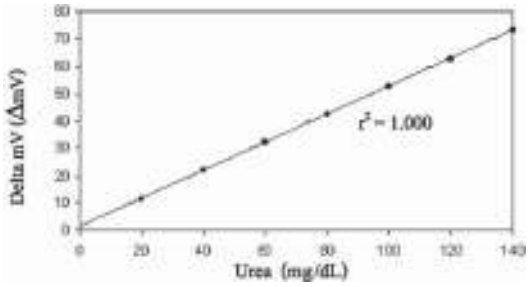

Figure 4 Calibration plot for estimation of serum urea using the enzyme biosensor electrode.

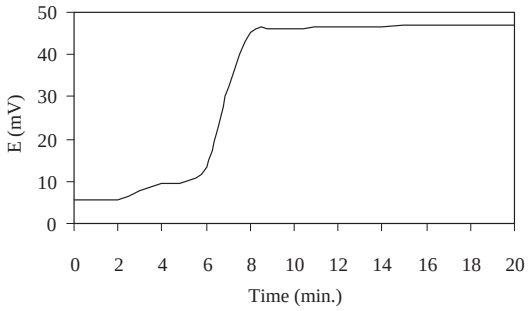

Figure 5 Typical response time curve urea electrode biosensor (0.01 M urea at pH 7.3)

CONCLUSION

The immobilized urease obtained from Jack bean described in this study was shown to be superior in several potential uses. Gelatin was an adequate material for urease immobilization to prepare the potentiometric urea biosensors. The characteristic response of this urea biosensor depended on the method of enzyme immobilization. The use of gelatin immobilized urea biosensor by chemical bond followed by cross-linking with dilute aqueous glutaraldehyde solution, showed the best response. Moreover, the biosensor electrode has fairly good sensitivity including long lifetime (more than 2 months) and the good reproducibility for blood serum samples.

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