Amperometric Determination of Urea Using Enzyme-Modified Carbon Paste Electrode

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An amperometric biosensor based on carbon paste electrodes (CPEs) for the determination of urea was constructed by enzyme (urease/GL-DH)-modified method. Urea was hydrolyzed to NH_4^+ by catalyzing urease onto the enzyme-modified electrode surface in sample solution. In the presence of α-ketoglutarate and reduced nicotinamide adenine dinucleotide(NADH), a liberated NH_4^+ produce to *L*-glutamate and NAD^+ by *L*-glutamate dehydrogenase (GL-DH). After the chemical reaction was proceeded, the electrochemical reaction was occurred that an excess of the NADH was oxidized to NAD^+. The oxidation current of NADH was monitored at +1.10 volt *vs.* Ag/AgCl. An optimum conditions of biosensor were investigated: The optimum pH range for catalyzed hydrolysis reaction of urea was pH 7.0-7.4. The linear response range and detection limit were 2.0×10^{-5}~2.0×10^{-4} M and 5.0×10^{-6} M, respectively. Another physiological species did not interfere, except *L*-ascorbic acid.

Key Words : Urea, Enzyme-modified carbon paste electrode

Introduction

Urea is widely distributed in nature, and its analysis is considerable interested in clinical and agricultural chemistry.[1,2] The most of enzymatic method for the determination of urea utilizes the catalyzed hydrolysis of urea.[3-7] The assay of urea can be followed by many different techniques such as colorimetry,[3] fluorometry,[4] and electrochemistry.[7]

Most of enzyme-modified electrodes[8-12] for urea have been used to potentiometric method. But, potentiometric methods using an urease have several disadvantages such as slow-response time and interference effect by the Na^+ and K^+ ions. Therefore, Adams[11] *et al.* assayed urea by colorimetric method using an immobilized enzyme reactor, and Kirsten[12] *et al.* developed an amperometric sensor for urea by measuring the current from the electrochemical oxidation of hydrazine. Seo[13] *et al.* determined NH_4^+ by amperometric method, using immobilization of *L*-glutamate dehydrogenase on the Immobilon-AV affinity membrane.

Recently, the incorporation of enzyme into a carbon paste was a valuable technique in the construction of amperometric enzyme electrode.[16] The enzyme-modified carbon paste electrode will become a great analytical techniques because of good specificity, sensitivity, simplicity.[14,15] In this paper, we will be report the development of an enzyme-modified carbon paste electrode incorporating an urease, and GL-DH into carbon paste for amperometic urea determination.

Experimental Section

Instrumentation and apparatus. Cyclic voltammetric experiments and steady-state measurements were carried out with a Bioanalytical Systems Model BAS 100B voltammetric analyzer interfaced to PC-IBM compatible computer. The pH measurement performed worked with a Orion 920A (Orion, USA). The platinum wire (Model MW 1032, BAS) and Ag/AgCl (sat'd KCl, Model MF 2063, BAS) were used as the auxiliary and reference electrode, respectively.

Materials. The source of all chemicals used for experiments are given in parentheses: nicotinamide adenine dinucleotide (NADH, Sigma); α-ketoglutarate (Sigma), *L*-glutamate dehydrogenase (GL-DH, EC 1.4.1.3., Sigma], urease (EC 3.5.1.5., Sigma), Urea (Sigma), Na_2HPO_4 (Merck), NaH_2PO_4 (Merck); and interferences such as *L*-asparagine, *L*-serine, *L*-glutamine, *L*-alanine, *L*-glutatione, *L*-phenylalanine, *L*-threonine, pyruvate, ascorbic acid, uric acid and cystine were purchased from Sigma Chemical Co..

Prepration of enzyme-modified carbon pastes electrodes. The enzyme-modified carbon pastes were prepared by thorough mixing of 0.1 mL of 1,000 units/mL urease and 0.1 mL of 1,000 units/mL GL-DH with graphite powder in a mortar (50%, v/v). An electrode body was made by tip of 1 mL polyethylene syringe (5 mm i.d.) cut off. The prepared mixed paste was packed under pressure into the syringe barrel. Electric contacts were made by thin copper wire through a plunger. Fresh electrode surfaces were obtained by squeezing out a small amount of the paste, and scraping with paper until the surface show a shiny appearance.

The electrode surfaces were subsequently rinsed with deionized water. Before all the experiments, the electrode surface was activated with anode scanning from 0.0 V to +1.3 V range.

The enzyme-modified electrodes were allowed to dry at room temperature for 2-3 hours, and were kept in 0.1 M phosphate buffer solution at refrigerator (4 °C), when not in use.

Measurement procedure. A constant potential of +1.10 volt *vs.* Ag/AgCl (Sat. KCl) was applied to the enzyme-modified electrode. The responses of electrode were measured for the sample solution added to 10 mL of 0.1 M phosphate buffer solution at 20 °C. In order to determine the

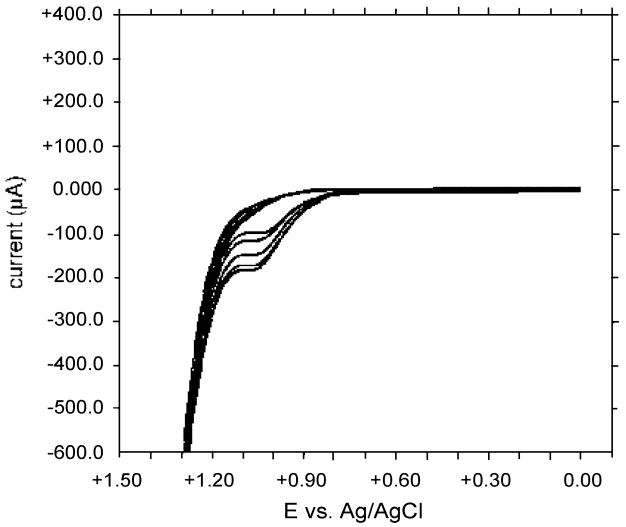

Figure 1. A series of cyclic voltammograms were obtained at an enzyme-modified carbon paste electrode: the peak height decreased as the urea added.

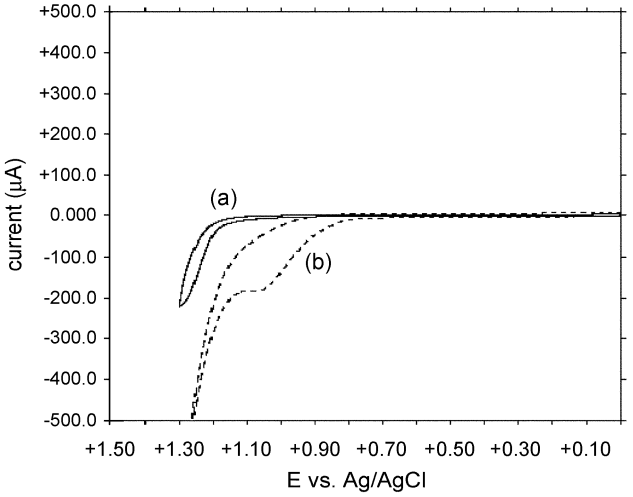

Figure 2. Cyclic voltammograms of an enzyme-modified carbon paste electrode in 0.1 M phosphate buffer (pH 7.2) contained 4.0×10^{-1} M α-ketoglutarate. 2.0×10^{-1} M urea.: (a) no NADH (b) in exist 4.0×10^{-4} M NADH.

optimum experimental conditions for determination of urea, amounts of NADH and enzymes, pH, interference effect, lifetime, and calibration curve were investigated.

The stock solutions of 1.0×10^{-2} M α-ketoglutarate, 5.0×10^{-3} M NADH, and 1.0×10^{-2} M urea were prepared in 0.1 M phosphate buffer solution. When not in use, stock solutions of NADH and α-ketoglutarate were stored in refrigerator (4 °C). At first, the sample solution contained 4.0×10^{-1} M α-ketoglutarate, 4.0×10^{-1} M NADH and 2.0×10^{-1} M urea. And, all sample solutions were thoroughly deaerated with nitrogen gas and continuous streams of nitrogen was passed over the solution while measurements were being taken.

The analytical signal was displayed as a series of peaks on the recorder (Fig 1). The currents obtained from the measurement is that the peak height gradually decreased as the urea added. Therefore, we confirmed the peak current decay was related to the concentration of urea. All data were shown the average value of three times measurements.

Results and Discussion

Electrochemical behaviors of the enzyme-modified CPEs. To evaluate the electrochemical characteristics of the enzyme-modified carbon paste electrode, a couple of cyclic voltammograms (CVs) were obtained at a enzyme-modified CPEs (Fig. 2). The voltammtric scanning range was 0.00 V +1.30 V *vs.* Ag/AgCl.

In the presence of 4.0×10^{-4} M NADH, 4.0×10^{-4} M α-ketoglutarate, 2.0×10^{-4} M urea, a clear bioelectrocatalytic oxidation wave was observed at +1.10 V *vs.* Ag/AgCl. In addition, the peak height gradually increased as the scan rate increased, suggesting the currents obtained from the measurements were diffusion controlled. Accordingly, all experiments were done at 100 mV/sec scan rate to obtain better-defined peaks.

The urea is hydrolyzed by urease, yielding two equivalents

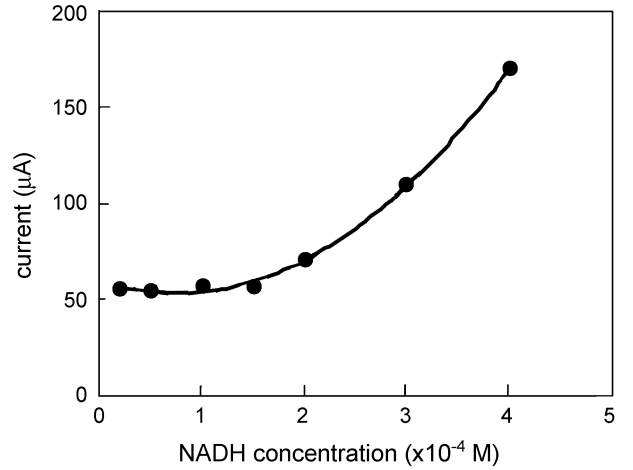

Figure 3. NADH-dependence of the coupled enzymatic reaction for enzyme-modified carbon paste electrode: 4.0×10^{-4} M α-ketoglutarate. 2.0×10^{-4} M urea.

of NH_4^+ ions per mole. In the presence of H^+ ions, reduced nicotinamide adenine dinucleotide (NADH), *L*-glutamate dehydrogenase (GL-DH), and NH_4^+ were reacted with α-ketoglutarate to yield *L*-glutamate and oxidized NAD^+. Concentration of urea was measured base on the urease catalyzed hydrolysis of urea; the α-ketoglutarate reacted with liberated NH_4^+ in the presence of reduced nicotinamide adenine dinucleotide (NADH) and *L*-glutamate dehydrogenase (GL-DH). The following three reactions would occur sequentially in the proposed mechanism.[18]

$$\text{urea} \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- \quad (1)$$

$$2NH_4^+ + \alpha\text{-ketoglutarate} + NADH \xrightarrow{\text{GL-DH}} L\text{-glutamate} + NAD^+ \quad (2)$$

$$NADH \rightarrow NAD^+ + 2e^- \quad (3)$$

After chemically reactions were equilibrium, we measured the current change that an excess NADH is oxidized to

NAD^+. This reaction was completed within a short time at the optimum concentration of NADH.

Figure 3. shows that the change in current with NADH concentration was measured in order to determine the optimum concentration range of NADH. As shown in Figure 3, the optimum limiting concentration of NADH was 2.0×10^{-4} M for 4.0×10^{-4} M α-ketoglutarate and 4.0×10^{-4} M urea, respectively. This result was probably due to chemical equivalent. So, the optimum concentration of NADH was needed at least equal to that of urea.

Electrode composition. The optimum amounts of the enzymes (urease/GL-DH) were investigated by altering the ratio of the enzymes (urease/GL-DH) to the carbon paste mixture, for 10 mL of sample solution containing 2.0×10^{-4} M urea, 4.0×10^{-4} M NADH and 4.0×10^{-4} M α-ketoglutarate, respectively (Fig. 4). The enzymatic reactions take place most effectively when the volume ratio of the enzymes

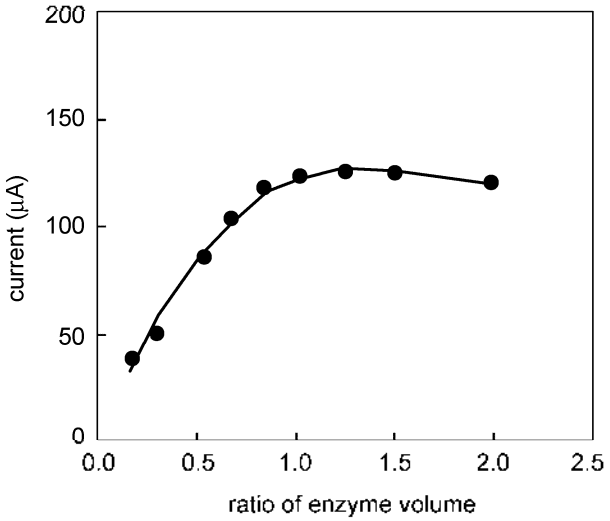

Figure 4. Effect of the enzyme ratio of enzyme-modified carbon paste electrode (urease/GL-DH) on the enzymatic reaction: 4.0×10^{-4} M NADH, 2.0×10^{-4} M urea, 4.0×10^{-4} M α-ketoglutarate.

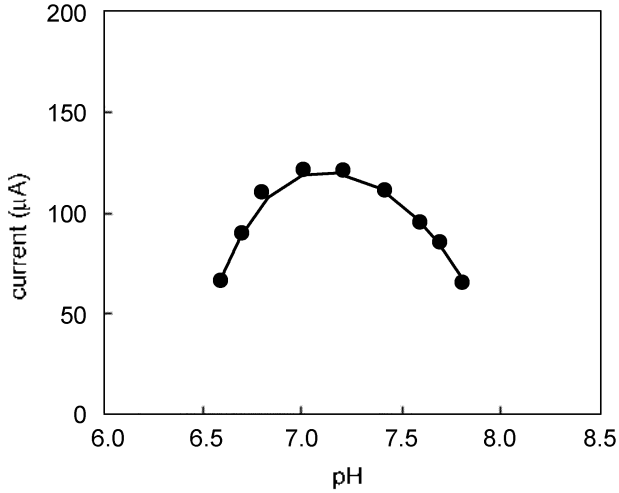

Figure 5. Effect of pH on the enzymatic reaction of enzyme-modified carbon paste electrode in 0.1 M phosphate buffer: 4.0×10^{-4} M NADH, 2.0×10^{-4} M urea, and 4.0×10^{-4} M α-ketoglutarate.

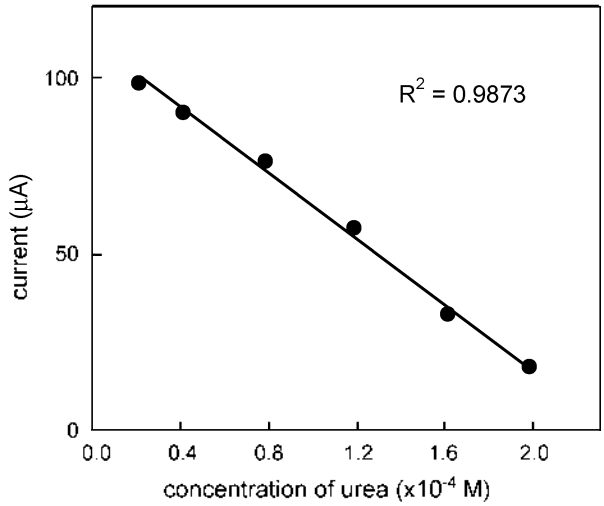

Figure 6. Calibration curve for urea determination with enzyme-modified carbon paste electrode in 0.1 M phosphate buffer (pH 7.2).

(urease/GL-DH) was between 0.8 and 2.0 as shown in Figure 4. The current increased with an increasing amount of enzyme up to 1.0 and above 2.0 it decreased. Hence, we used 1 : 1 volume composition to construct the enzyme-modified electrode: 0.1 mL of urease (1,000 units/mL)/0.1 mL of GL-DH (1,000 units/mL).

pH. The effect of pH for the electrode response was investigated using 0.1 M phosphate buffer (Fig. 5). The pH optimum is 7.3 on phosphate buffer,[19] whereas GL-DH has a pH optimum of approximately pH 8.0[20] in according to the previous report. In this study, the coupled enzymatic catalyzed hydrolysis reaction of urea occurs affirmatively at pH 7.0-7.4 using 0.1 M phosphate buffer solution, therefore the pH 7.2 was taken for the measurement of urea.

Determination of urea. A typical calibration curve of urea using enzyme-modified carbon paste electrode included urease and *L*-glutamate dehydrogenase (GL-DH) was shown in Figure 6. We determined urea by measuring the current change that the excess NADH is oxidized to NAD^+. Thus, NADH oxidation currents were measured with change of urea for 10 mL of sample solution containing 2.0×10^{-5} M to 2.0×10^{-4} M (R^2=0.9873) urea (Fig. 6). The detection limit was 5.0×10^{-6} M. These results are comparable to other report.

Interference materials. In the presence of 4.0×10^{-5} M urea, interference effects were investigated by measuring the responses of the electrode to 4.0×10^{-5} M and 4.0×10^{-4} M of *L*-asparagine, *L*-serine, *L*-glutamine, *L*-alanine, *L*-glutatione, *L*-phenylalanine, *L*-threonine, pyruvate, ascorbic acid, uric acid and cystine, respectively (Table 1). These materials give a relative error of less 2% in the current obtained by amperometric method (+1.10 *vs.* Ag/AgCl).

Precision and accuracy. The precision was studied by measuring the three times for urea analysis. Table 2 summarizes the repetitive measurement of urea concentration. The average standard deviation was ± 0.3(μA). These results are better than Lee's result[22] for the continuous automated determination of urea by a potentiometric method, and also Adam's[11] using coulometric flow analysis.

Table 1. Effect of interference materials on the determination of urea with enzyme-modified carbon paste electrode

Interference materials	Concentrations			
	4.0×10^{-5} M[a]		4.0×10^{-4} M[b]	
	I (μA)	Error (%)	I (μA)	Error (%)
urea	92.0	-	92.0	-
L-asparagine	91.0	-1.09	91.0	-1.09
L-serine	91.5	-0.54	91.0	-1.09
L-glutamine	92.0	0.00	92.0	0.00
L-alanine	91.0	-1.09	90.0	-2.17
L-glutatione	92.0	0.00	91.0	-1.09
L-phenylalanine	93.0	+1.10	92.0	0.00
L-threonine	92.0	0.00	92.0	0.00
pyruvate	92.0	0.00	92.0	0.00
ascorbic acid	91.0	-1.09	91.0	-1.09
uric acid	91.0	-1.09	90.0	-2.17
cystine	92.0	0.00	92.0	0.00

Table 2. Precision test for enzyme-modified carbon paste electrode for urea

Concentration (M)	Current (μA) (x + s)[a]	Rel. Std. Dev. (%)
5.0×10^{-5}	93.0 ± 2.8	3.01
7.5×10^{-5}	62.0 ± 2.6	4.19
1.0×10^{-4}	55.0 + 2.4	4.36
2.0×10^{-4}	35.0 ± 1.6	4.57

x: average of current, s: standard deviation. [a]three assays

Table 3. Accuracy test for enzyme-modified carbon paste electrode of urea

Urea (M)		Recovery (%)	Rel. errror (%)
Added	Found[a]		
5.0×10^{-5}	5.11×10^{-5}	102.2	+2.2
7.5×10^{-5}	7.51×10^{-5}	100.1	+0.1
1.0×10^{-4}	1.01×10^{-4}	101.0	11.0
2.0×10^{-4}	2.05×10^{-4}	102.5	12.5

a: x + s − 101.5%, x: average of recovery, s: standard deviation

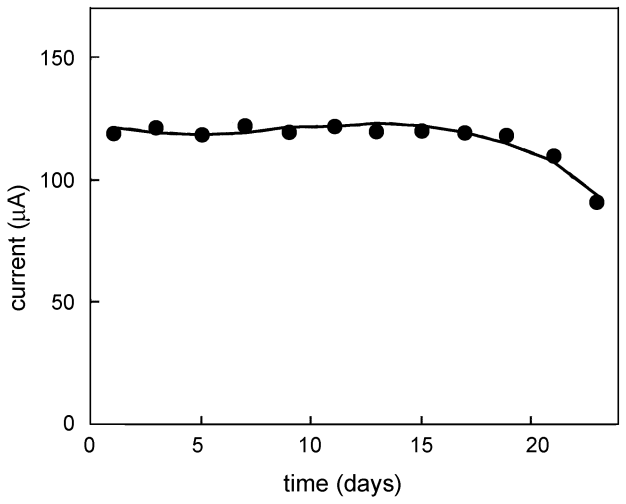

Figure 7. Life time of the enzyme-modified carbon paste electrode.

And, Table 3 shows the accuracy test for the determination of urea by the amperometric method. The average recovery was 102% (1.7%) comparable to data of Lee's (99.6%) and Adams' (100%).

Table 4. Comparison study of present method with AOAC method for the urea using the enzyme-modified carbon paste electrode

Sample of number	Urea (mg/dL)		Recovery (%)
	Present method	AOAC method	
sample 1	16.5	16.7	98.8%
sample 2	15.0	15.2	98.7%
sample 3	11.2	11.0	101.8%

Stability. The stability of carbon paste electrodes was investigated for a sample solution containing 2.0×10^{-1} M urea, 4.0×10^{-1} M NADH, and 4.0×10^{-1} M α-ketoglutarate (Fig. 7).

Three times measurements per day were carried out two consecutive days. When the electrode was not used, it was stored at 4 °C. The response of enzyme-modified carbon paste electrode was stable for 15 days. After 20 days, the activity of the sensor was found to be 71% of its original value. This is probably due to activity loss of enzyme-modified electrode.

Comparison study. The determination of urea in human blood was carried out for three samples to accomplish comparison study. The results were compared with those given by AOAC method.[24] The agreement was very well; The recovery from the three samples ranged from 98.7%-101.8%.

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