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Development of enzyme sensors based on the gas permeation with the use of a hollow fiber membrane

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ABSTRACT

A novel enzyme sensor based on the gas permeation with the use of a hollow fiber membrane was developed and the device was applied to flow-injection analysis (FIA) of urea with an acid urease column as a recognition element. A flow-type of the biosensing system used in this study was as sembled with a sample injection valve, a gas-diffusion device with an immobilized acid urease, and a flow-through cell attached to a UV/VIS detector. The gas-diffusion device has a double tubing structure. The inner tubing was gas permeable tubing which was a hollow fiber membrane, and bromothymol blue as a coloring agent was pumped through in this tubing. The outer tubing was fused silica capillary for flowing carrier solution and a monolithic silica column was constructed between inner and outer tubing. An acid urease was immobilized by using of glutaraldehyde crosslinking method onto the silica monolith followed by alkylamination of the constructed mono lithic silica. Standard urea solutions were measured through monitoring variations in absorbance resulting from pH shift due to CO_2 molecules enzymatically generated in acidic condition. A wide, linear relationship was obtained between the concentration of urea (1.25–1,000 μM) and the change in absorbance. This FIA systemgave higher sensitivity than that previous system did (25–500 μM). The proposed FIA system was applied to determination of urea in skin toners as real samples.

Keywords: Acid urease; Enzyme sensor; Flow-injection analysis; Hollow fiber; Silica monolith

1. Introduction

Enzyme sensor is widely used in the world for determination of the substrate. The principle of the enzyme sensors is detection of the enzymes reactions with their substrates. There are many enzymes, which produce gases as a product. For example, urease [1,2], L-lysine oxidase [3], histamine oxidase [4], arginine deiminase [5] produce ammonia by their reaction. And urease [1,2], phenylalanine decarboxylase [6], L-tyrosine decarboxylase [7], oxalacetate decarboxylase [8] produce CO_2. Therefore, if we obtained the good analytical method for their gases, the method will be applied to the enzyme sensor, which produced the gas.

In this study, we developed novel gas-diffusion device based on the follow fiber membrane and the system was applied to a determination of urea concentration in real samples.

Analysis of urea has been extensively investigated thought urea is one of useful chemicals and used as fertilizer [9,10], feed [11,12], raw materials of synthetic resins [13,14], cosmetics [15–17] and so on. There are so many reports on flow-injection techniques and all of those enzymatic methods for urea are using urease

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from jack bean [18–20], of which optimal pH is located in a narrow range of 7.0–8.0 and are based on the measurement of the pH change or ammonia released from enzymatic hydrolysis of urea. The catalytic hydrolysis of urea by urease as follows:

$$\mathrm{Urea} + 2\mathrm{H_2O} + \mathrm{H^+} \xrightarrow{\text{Urease}} 2\mathrm{NH_4^+} + \mathrm{HCO_3^-}$$

If the enzyme can catalyze an urea in lower pH region such as 3.0 or 4.0, the product (carbon dioxide) in the enzyme catalyzed reaction will not exist as HCO_3^- but CO_2.

We have investigated a new simple FIA system for a determination of urea concentration based on a CO_2 detection with use of acid urease [21]. In this study, we developed a novel gas-diffusion device in which a monolithic silica column as a support for immobilization of acid urease was constructed and their performance characteristics were investigated.

2. Experiments

2.1. Materials and reagents

Acid urease (from *Lactobacillus fermentum*) containing 95% lactose, NAGAPSHIN, was kindly provided by Nagase & Co., Ltd. (Osaka, Japan) and was purified by ultrafiltration [22,23]. A hollow fiber membrane, poly-4-methyl-1-pentene (inner diameter: about 190 μm, outer diameter: about 250 μm) was kindly obtained from Dainippon Ink and Chemicals, Inc. (Tokyo) and was used as a gas permeability membrane. A gas-diffusion device consists of a double tubing structure (inner tubing was the hollow fiber membrane and outer tubing was fused silica capillary (GL science)). As a support for immobilization of acid urease, monolithic silica column was constructed between the hollow fiber membrane and the polytetrafluoroethylene (PTFE) tubing. Bromothymol blue (BTB; water soluble) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo). Urea (biochemical grade) and BTB (water soluble) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were commercially available and of analytical grade. Ultrapure water with a resistivity of 18.2 MΩ−cm was obtained with an EQG-3S system (Nippon Millipore K. K., Tokyo), and used in all procedures.

2.2. Gas-diffusion device

A microfluidic gas-diffusion device was developed in this study and its schematic illustration was shown in Fig. 1. The gas-diffusion area of the device had a double tubing structure. At an upstream of the device, a PTFE tubing for carrier solution was connected by a T-type connector 1 with another PTFE tubing which was outer tubing of gas-diffusion area. Then, the carrier was wasted via PTFE tubing connected by a T-type connector 2. The hollow fiber membrane used as a gas permeable tubing was put it in the fused silica capillary. A PTFE tubing as a flow line of BTB solution was connected with the follow fiber membrane by the connector 1. The flow line between the connector 1 and T-type connector 1 had triple tubing structure to fit the connector. At the down stream of the device, the follow fiber membrane was connected with PTFE tubing with the connector 2. The flow line between the T-type connector 2 and connector 2 also had triple tubing structure. The BTB solution absorbing CO_2 gas in the carrier solution by across the follow fiber membrane at the gas-diffusion area of the device was pumped through the UV/VIS detector, and the change in absorbance was monitored.

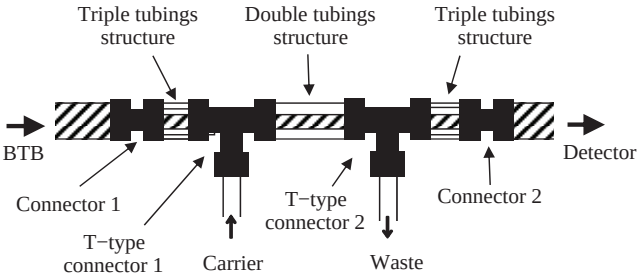

Fig. 1. Schematic illustration of gas-diffusion device.

2.3. Immobilization of acid urease onto constructed silica monolith

A 54 mg polyethylene glycol, 0.2 mL tetramethoxysilane and 10 mM acetic acid solution (0.2 mL) was mixed in a microtube, and the mix solution was shaken for 30 min at 4°C. Thereafter, the solution was injected into a fused silica capillary having a double tubing structure, and the capillary was allowed to stand for 24 h at 40°C. A solution of 0.2 M ammonia solution was injected into the capillary for rinse. After then, 10% γ-APTES (aminopropyl triethoxysilane, pH 3.45) was treated prepared silica monolith for 3 h at 75°C. This alkilaminated silica monolith was activated by injection of 2.5% glutaraldehyde solution for 1 h. Finally, 100 mg/mL acid urease solution was injected into the monolithic column and coupled with the silica monolith for 24 h.

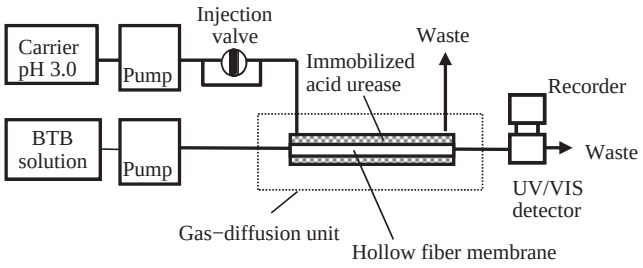

Fig. 2. Schematic illustration of FIA system used in this study.

2.4. Flow system and procedure

A schematic diagram of the flow system is shown in Fig. 2. The system was assembled with a Two double-plunger pump (PU-970, JASCO Corp., Tokyo) for carrier solution and coloring agent, a rotary injection valve equipped with a 20 μl sample loop (Syringe Loading Sample Injector 7120, Rheodyne Inc., California, U.S.A.), gas-diffusion unit on which acid urease was immobilized (Fig. 1), a UV/VIS detector (UV-970, JASCO Corp., Tokyo) with a flow-through cell (volume 32 μl, light-path length 10 mm), and a pen recorder (Multi-Pen Recorder; type R-62M3, Rikadenki Kogyo Co. Ltd., Tokyo). The flow rate of carrier (25 mM Gly-HCl buffer, pH 3.0) and coloring reagent (50 μM BTB, pH 7.6) were 0.4 mL/min, respectively.

Gly-HCl buffer as the carrier solution was successively pumped through the system. Sample solutions (20 μL) were introduced into the system via the rotary injection valve. Carbon dioxide formed in the enzymatic hydrolysis of urea was transferred to the gas-diffusion device consisting of a double tubing structure. The absorbance of BTB flowing streams in the ultrathin follow fiber membrane was varied by gaseous CO_2 diffusion across the membrane, and subsequent decrease in absorbance at 617 nm due to the reaction was successively monitored by a flow-through type of a UV/VIS detector and displayed on the pen recorder. The coloring reagent and carrier solution were passed with a wet nitrogen streaming (120 mL/min) into the reservoir. Twenty millimolar urea solutions were prepared in 25 mM Gly-HCl buffer (pH 3.0) and then diluted (from 100 to 1.0 μM) with the same buffer. The solution was used for standard urea solution for investigation of determination of urea with use of proposed system.

2.5. Influence of internal ammonia and CO_2 on the determination of urea using the FIA system

The influence of endogenous ammonia on the determination of urea was evaluated by injecting ammonium chloride containing a urea solution into the FIA system. Also, the influence of CO_2 on the analysis was evaluated by injecting 500 μM $NaHCO_3$ solutions followed by heat treatment of the sample in the thermostat bath at several temperatures.

2.6. Determination of urea in skin toners

Several kinds of commercially available skin toners were diluted with distilled water or with urea-containing water, and then injected into the FIA system to determine urea by the standard addition method.

An F-kit method was used for validation. Urea in skin toner was hydrolyzed to form ammonia and carbon dioxide in the presence of urease contained in the kit. The liberated ammonia reacted with 2-oxoglutarate to produce L-glutamate in the presence of glutamate dehydrogenase, which was also contained in the kit, and also reduced a type of nicotinamideadenin dinucleotide (NADH). The amount of NADH oxidized in the above reaction was stoichiometrically equal to the amount of ammonia. NADH was determined by means of its absorbance changing at 340 nm. From the above principle, the concentrations of urea in skin toners were determined based on the above-mentioned principle. A practical method of the F-kit was referred to the manual.

3. Results and discussion

3.1. Evaluation of the immobilized acid urease activity

An activity of the prepared acid urease column (covalently immobilized onto the silica monolith) was compared with that of acid urease absorbing silica monolith. Both enzyme columns catalyze urea and show a same activity. However, the activity of acid urease absorbing column was decrease to about 50% for 1 week. On the other hand, the activity of the covalently immobilized acid urease column was not decrease for 3 months (data not shown). Therefore, this covalently immobilized acid urease silica monolithic column was used in this study.

3.2. Determination of urea concentration

Determination of urea by using immobilized acid urease integrated into microflow gas-diffusion device based on a CO_2 detection was investigated.

Each of 20 μl of various concentrations of urea solutions was injected into this FIA system, and CO_2 molecules liberated in the enzyme-catalyzed reaction were measured. Fig. 3 was comparison of response curves of proposed FIA system and conventional FIA system of which acid urease was immobilized onto CPG

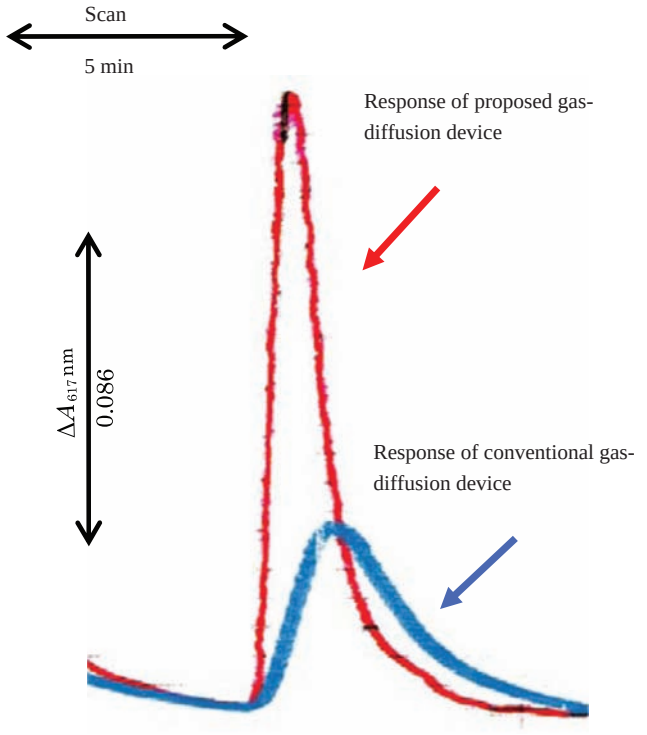

Fig. 3. Comparison of response curves between proposed and conventional gas-diffusion device.

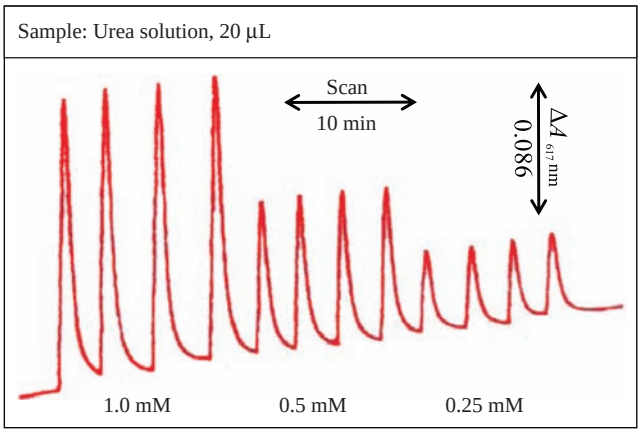

Fig. 4. Response curves to 20 mL injections of urea standards with various concentrations.

(controlled pore glass) and of which column was introduced upstream of the gas-diffusion device. As shown in Fig. 3, response curves obtained from proposed system was more sharp and higher than that obtained from conventional system. This result shows that proposed system was sensitive because of the diluteness of the enzymatic produced CO_2.

As shown in Fig. 4, sharp peaks were reproducibly obtained though the base line was slightly increased,

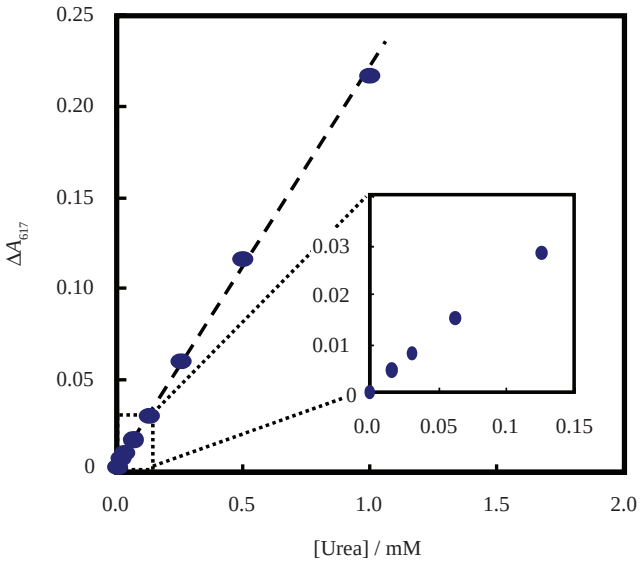

Fig. 5. Comparison of response curves between proposed and conventional gas-diffusion device.

and one assay of a sample injection took 4 min or shorter. The peak height with use of this gas-diffusion device (change in absorbance) was a much higher than the peak height with use of conventional gas-diffusion device, though injection volume in this study was one-fifth (data not shown). This result showed the system was sensitive because the thickness and the surface area against flowing value of the gas permeable membrane were superior to conventional one from the point of gas diffusion.

As shown in Fig. 5, with the increasing concentration of urea solutions, variation in the absorbance increases and then tends toward a constant value. A good linearity was obtained in a range of 1.25 μM–1.0 mM and the correlation coefficient was calculated to be 0.996 and the relative standard deviation (r.s.d.) for urea determination with each concentration was below 3.0% ($n = 5$).

3.3. Influence of ammonia on the response of the FIA system

To investigate the influence of ammonia on the urea determination, several concentrations of urea solution containing 10 mM ammonium chloride were injected and the absorbance was compared with same concentrations of urea solution without ammonium chloride ($n = 5$). As shown in Fig. 6, the absorbance of urea solution was not different from those of urea solution including 10 mM NH_4Cl. The results mean that this method should not suffer from ammonia, even in the concentration of containing ammonia was 100-fold higher than that of urea. Therefore, this system based

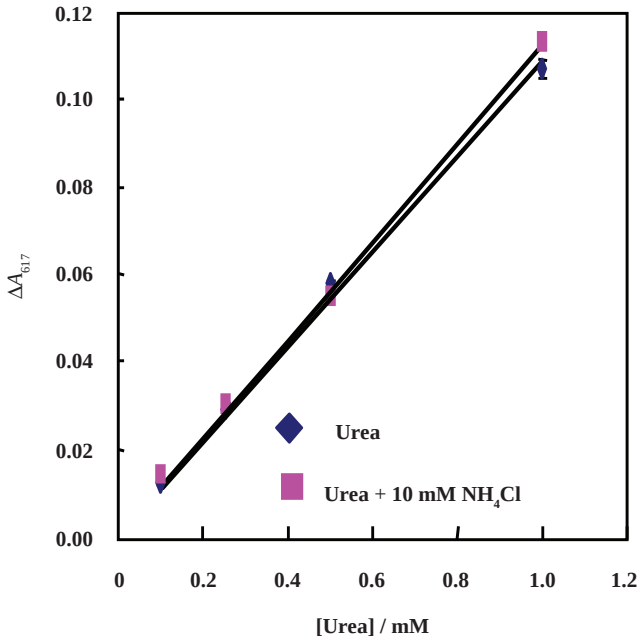

Fig. 6. Response curves to 20 mL injections of urea standards with various concentrations.

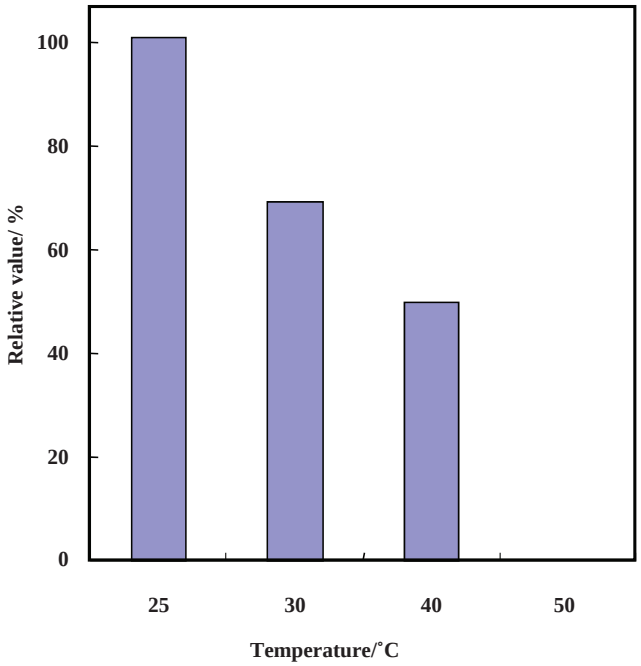

Fig. 7. Removal of endogenous CO_2 in the sample solution by heat treatment in the thermostat bath.

on CO_2 detection would have good advantage over any conventional systems based on NH_3 detection.

3.4. Removal of endogenous CO_2 in the sample solution by heat treatment in the thermostat bath

Endogenous CO_2 in the sample solution must interfere correct determination of urea by using urease based on CO_2 detection. And much CO_2 was dissolved in the sample solution from the atmosphere. Therefore, removal of endogenous CO_2 in the sample solution was very important.

A 0.5 mM $NaHCO_3$ solution followed by heat treatment in the thermostat bath at the several temperatures was injected into the system as an internal CO_2, and the influence of the solution on the response of this system was evaluated. The result is indicated in Fig. 7. When the 0.5 mM $NaHCO_3$ solution which was treated by 25°C for 5 min was injected, very large peak caused by CO_2 was observed. As the temperature of the sample treatment was higher, the peak was smaller. When the 0.5 mM $NaHCO_3$ solution was treated at 50°C for 5 min, no signal was detected. This result indicates that the internal CO_2 will be able to removed complementally.

3.5. Determination of urea in real samples

From these results, we considered this FIA system might be applicable to determination of urea concentration in real samples. So, the FIA system was applied

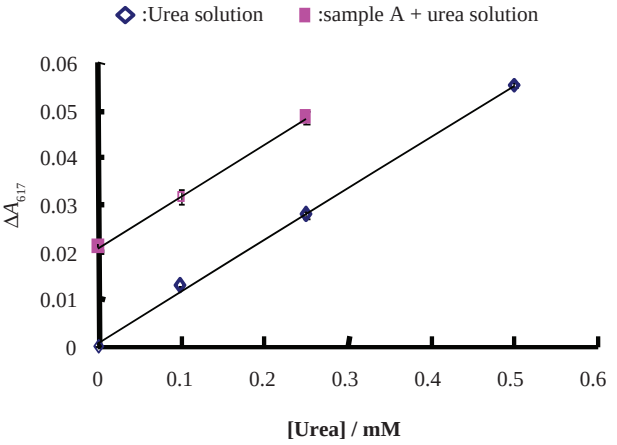

Fig. 8. Determination of urea in a real sample by standard addition method.

to determine urea in five kinds of real skin toners by using standard addition method. As shown in Fig. 8, the absorbance when sample A was injected into the FIA system increased as concentration of adding urea increased ($n = 3$). A good recovery ratio which was 101.6%, and good relative ratio with conventional F-kit method which was used for determination of urea were obtained. Therefore, we applied the FIA system to determination of urea concentration in the other real samples.

Table 1
Determination of urea in real samples and comparison of the value with F-kit method.

Products	Recovery ratio/%	FIA/mM	F-kit/mM	Relative ratio/%
Sample A	101.6	468	444	105.3
Sample B	95.1	376	403	93.3
Sample C	103.4	7.88	7.78	101.4
Sample D	100.9	18.4	17.9	102.9
Sample E	101.9	504	490	103

From the results of five kinds of skin toners, the recovery ratios calculated with the standard addition method ranged from 95.1% to 103.4%. Concentrations of urea in real samples evaluated by this method were compared with those evaluated by the F-kit method (Table 1). This result shows that a good relative ratio between concentrations of urea by using the proposed FIA method and that of F-kit method was obtained.

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