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Development of a Removal System for Ammonia with Use of an Immobilized Enzyme Column

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A removal system for ammonia from analytical samples in a FIA (Flow-Injection Analysis) system was developed using an immobilized glutamate dehydrogenase (GLDH) column. GLDH is one of oxidoreductases, which catalyzes NADH-dependent conversion from 2-oxoglutarate and ammonia to L-glutamate. This enzyme was covalently immobilized onto porous glass beads. The immobilized GLDH beads were packed into a small polymer column and then mounted in a water-jacked holder. A removal system was assembled with a plunger pump, a rotary injection valve with a sample loop, the GLDH column unit, a gas diffusion unit, a flow-through type of a UV/VIS detector, and a pen recorder. To investigate the effect of the GLDH column on removal of ammonia, ammonia solutions with various compositions were injected into this system. The optimal concentration of NADH and 2-oxoglutarate for determination of ammonia with this system was 0.6 mM and 10 mM, respectively. In a case of removal of ammonia, the higher concentration of the both substances were, the higher removal effect was observed. The amount of ammonia removed using this system was up to 0.5 mM (injection volume: 0.1 ml, flow rate: 0.4 ml/min). This catalytic activity for removal of ammonia solutions was kept constant during 3 weeks observation.

Keywords: Flow injection analysis, ammonia, removal, glutamate dehydrogenase, determination of urea.

1. INTRODUCTION

Urea is one of the most important catabolite in amino acid metabolism and purine catabolism. The assay of urea in blood or in urine presents valuable information about the balance of protein ingest or the liver function. The analysis of urea is one of the most important subjects not only in the field of laboratory tests but also in many other analytical fields, because urea is used as a fertilizer [1, 2], feed [3, 4], cosmetics, medicine, raw materials of synthetic resins [5, 6]. Therefore, simple and useful method for urea determination has been demanded and investigated. In such analytical fields, many studies of the determination of urea have been based on the measurement of the pH change or ammonia released in hydrolysis of urea by using urease catalysis. There are many reports with flow injection analyses based on electrochemical methods such as amperometry [7, 8], potentiometry [9, 10], conductometry [11, 12], calorimetry [13], and optical methods such as absorptiometry [14], fluorescence spectrophotometry [15-18], emission spectrophotometry [19-21]. All of these enzymatic methods for urea ultilize urease from jack bean, of which optimal pH is located in a range of 7.0 – 8.0. On the other hand, we have reported the urea determination in rice wine by using not a urease but acid urease[22, 23]. Because urea is considered as a precursor of ethylcarbamate which has been known to be carcinogenic, teratogenic, and mutagenic, the monitoring of urea in rice wine has been required. In any way, since the principles of the system for determination of urea is based on measurement of ammonia produced by the enzyme-catalyzed reaction, so endogenous ammonia molecules in the sample results in interfering with the determination of correct concentration of urea. Glutamate degydrogenase (GLDH), one of the oxidoreductase, catalyzes ammonia with 2-oxoglutarate to L-glutamate. In the reaction, GLDH requires a NADH for catalysis. Therefore, immobilized GLDH column was used as a recognition element for ammonia determination in combination with flow injection photometry by monitoring change in absorbance of NADH [24, 25]. In this study, we used the GLDH column not as a sensing element but as a reactor for removal of endogenous ammonia, and the properties of GLDH column as the functional element for ammonia removing was investigated.

2. EXPERIMENTAL

2.1. Materials and reagents

Glutamate dehydrogenase (from Beef liver, EC 1.4.1.3) was obtained from Oriental Yeast Co., Ltd. (Tokyo). Controlled-Pore glass (CPG, mean pore diameter 24.2 nm, particle size 120 – 200 mesh) was purchased from Funakoshi Co., Ltd. (Tokyo). A gas diffusion device and porous PTFE (polytetrafluoroethylene) tubing (mean pore diameter 1.0 μm, inner diameter: 1 mm, outer diameter: 2 mm, length of part of gas diffusion layer: 120 mm) were purchased from F·I·A Instruments Co., Ltd. (Tokyo) and Flon Industry (Tokyo), respectively. Ammonium acetate, 2-oxoglutaric acid, and β-nicotinamide adenine

demucleotide disodium salt, reduced form ($NADH\text{-}2H_2O$) were purchased from Wako Pure Chemical Industies, 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 (Millipore, Tokyo).

2.2. Immobilization of glutamate dehydrogenase

Glutamate dehydrogenase was covalently immobilized onto alkylaminated CPG as described previously [26]. The alkylamninated glass beads (1.0 g) were activated with glutaraldehyde (2.5 %, 25 ml) under reduced pressure, and then, glutamate dehydrogenase solution (100 mg/ml, 9.0 ml) prepared in phosphate buffer (50 mM, pH 7.0) was coupled with the glass beads with shaking at 4 ℃ for 50 hours. The immobilized preparations were treated with 10 % sodium borohydrate solution that was freshly prepared by mixing with 0.2 M phosphate buffer (pH 5.0, 5.0 ml), 0.1 M NaH_2PO_4 (1.0 ml) and 4.0 ml pure water. The reducing process was repeated ten times and then washed with phosphate buffer (0.1 M, pH 7.0). The yield was calculated by measuring absorbance at 280 nm in the enzyme solutions before and after the coupling process. The immobilization yield was 89.6 %. The immobilized preparations were packed into a small polymer column and then mounted in a water-jacked holder.

2.3. Apparatus and flow system

Schematic diagrams of the flow systems used in this study were shown in Fig. 1. The FIA system shown in Fig.1-(A) was used for measurement of GLDH activity. On the other hand, the system of Fig.1-(B) was used for evaluating whether ammonium ions were removed or not.

The system shown in Fig.1 (A) was assembled with a double-plunger pump (Sanuki DM3M-2044, Sanuki Industry Co., Ltd., Tokyo), a rotary injection valve with a 100 μl sample loop, the immobilized acid urease column with a water-jacket, a UV/VIS detector (UV-970, JASCO Corp., Tokyo) with a quartz flow-through cell (volume 32 μl, light-path length 10 mm), and a pen

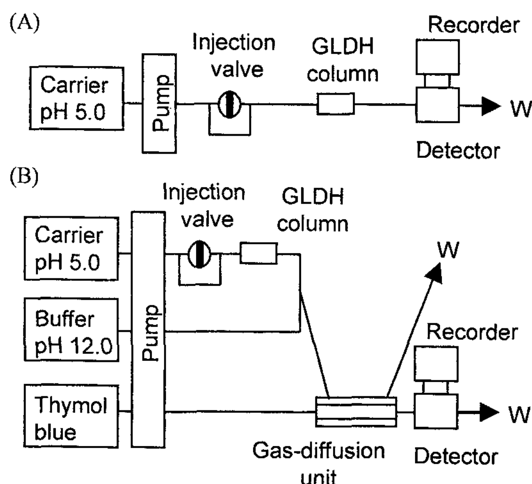


Fig. 1. System for measurement of a GLDH column activity (A) and a system for evaluation of removal ability of a GLDH column (B)

recorder (Multi-Pen Recorder; type R-62M3, Rikadenki Kogyo Co. Ltd., Tokyo). As shown in Fig.1 (B), for evaluation of ammonia removal, the double-plunger pumps for alkaline buffer (100 mM sodium phosphate, pH 12.0, 0.4 ml/min) and for coloring agent (0.15 mM Thymol Blue solution, pH 8.4, 0.8 ml/min) and the gas diffusion device with a constant temperature bath (F·I·A Instruments Co., Ltd.) were connected to the system shown in Fig.1 (A) The temperature around the gas diffusion unit was regulated with a constant temperature bath.

2.4 Evaluation of activity of the immobilized GLDH column.

Tris-HCl buffer (50 mM, pH 8.0) as the carrier solution (0.4 ml min^{-1}) was successively pumped through the system. Sample solutions containing NADH, 2-oxoglutarate, and ammonium acetate were introduced into the system via the rotary injection valve. The solutions containing equivalent amounts of NADH and 2-oxoglutarate without ammonium acetate were used as the control of the sample solutions. From the differences between the absorbance of sample solutions and that of the control solutions, activity of an immobilized GLDH column was evaluated.

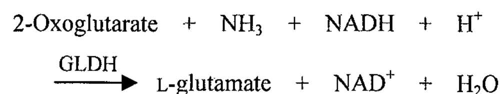
2.5 Investigation of removal of ammonium ions by the immobilized GLDH column

Same carrier solutions with the above procedure were used. Ammonium ions released in the enzyme catalysis were converted to gaseous ammonia molecules by mixing with the strongly alkaline buffer (gas-diffusion buffer: 100 mM sodium phosphate, pH 12.0), and the mixed solution (0.8 ml min^{-1}) was transferred to the gas-diffusion unit consisting of a double tubing structure [27]. The absorbance of Thymol Blue flowing streams in the PTFE tubing was varied by gaseous ammonia diffusion across the PTFE tubing, and subsequent increase in absorbance at 596 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 solution (Thymol Blue solution, 0.15 mM, pH 8.4, 0.8 ml min^{-1}) was passed with a wet nitrogen streaming (120 ml min^{-1}) into the reservoir.

3. Results and Discussion

3.1 Measurement of the activity of the immobilized GLDH column Transformation of NH_3 to L-glutamate by GLDH column is as follows:

$$\text{2-Oxoglutarate} + NH_3 + NADH + H^+$$



$$\xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^+ + H_2O$$

In the reaction, all of 2-oxoglutarate, NH_3, and NADH were substrates and were essential for the enzyme reaction. Therefore, the solution containing these three substrates was described as a substrates solution, and the substrates solution without ammonium acetate was used for the blank of the substrates solution. NADH was used for the enzyme catalysis and it had a strong absorbance at 340 nm. On the other hand, NAD^+ which was the

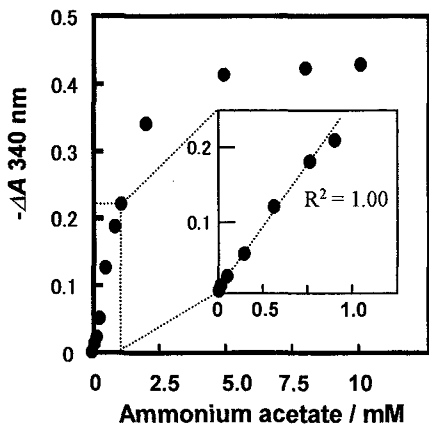


Fig. 2. Calibration graph for ammonium acetate

product in the reaction had a only weak absorbance at 340 nm. Because the enzyme activity for catalysis of ammonium ion's conversion to glutamate could be measured by monitoring the different absorbance between the substrates solution and the blank solution, the difference in absorbance (-ΔA) was used for the evaluation of activity of the GLDH column.

In order to measure the column activity, the substrates solution (0.3 mM NADH, and 10 mM 2-oxoglutarate with various concentrations of ammonium acetate) or without ammonium acetate (blank solution) were injected into the sensing system (Carrier: 50 mM Tris-HCl buffer (pH 8.0)). The differences in absorbance between substrates solutions and the blank solutions at 340 nm versus concentrations of ammonium acetate (0.04 mM – 10.0 mM) were shown in Fig.2. With the increasing concentration of ammonium acetate, the absorbance increases and then tends toward a constant value. The good linearity was obtained in a rage of 0.04 – 0.8 mM and the square of correlation coefficient was calculated to be almost 1.00. One assay took 5 min or shorter for the response to ammonium acetate.

3.2 Effects of pH on the column activity

To investigate the effect of pH on the activity of the GLDH column, substrates solutions (0.3 mM NADH, 10 mM 2-oxoglutarate, 1.0 mM ammonium acetate) with various pH values ranging from 4.5 to 9.0 in a step of 0.5 were injected into the sensing system. As shown in Fig. 3, under an acidic condition, immobilized GLDH column couldn't exhibit the activity sufficiently. On the other hand, in the neutral region of pH, the high activity was shown. These results mean that a significant amount of ammonia was catalytically removed by GLDH column in the neutral pH. The optimal pH for the GLDH column was to be 8.0.

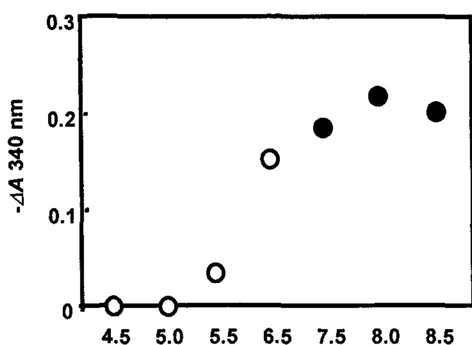


Fig. 3 pH profile of the GLDH column. O: Citrate buffer (50 mM) was used as a carrier solution, ●: Tris-HCl buffer (50 mM) was used as a carrier solution.

3.3 Effects of concentrations of the components of the substrates solution on the GLDH column activity.

Since the concentration of a substrate influences on the enzyme activity, the effect of the components of the substrates solution on the column activity was investigated. The substrates solutions (0.3, 0.6 mM NADH, 10, 50, 100 mM 2-oxoglutarate, and 0.5 mM ammonium acetate) were introduced into the sensing system. The calibration curves for ammonium acetate were shown in Figure 4. The absorbance of each constitution increased, as the concentrations of ammonium acetate were increased. With the increasing concentration of ammonium acetate, the effect of NADH was increased. Fig. 4 shows that the concentration of 2-oxoglutarate was preferred 10 mM to 50 mM to obtain a wide range for the determination of ammonium ions ($R^2 \geqq 0.99$). We considered the reason why the low concentration of a 2-oxoglutarate enabled to extend the linear range wider, that the absorbance of a 2-oxoglutarate should make the absorbance of the blank increase. The optimal substrate solution for determination of ammonium ions was the solution containing 0.6 mM NADH and 10 mM 2-oxoglutarate.

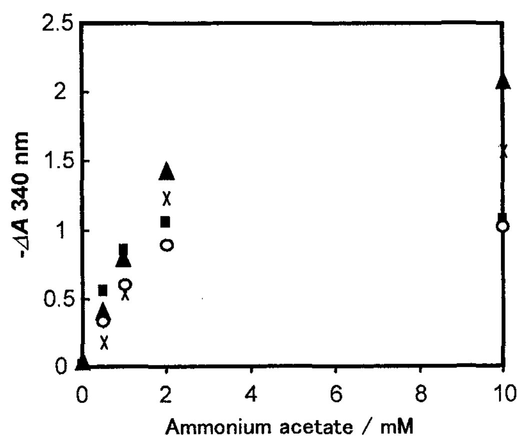


Fig. 4 Effects of concentrations of the components of the substrates solution on the GLDH column activity and the determination range. o: 0.3 mM NADH, 10 mM 2-oxoglutarate, ■: 0.3 mM NADH, 50 mM 2-oxoglutarate, ▲: 0.6 mM NADH, 10 mM 2-oxoglutarate, ×: 0.6 mM NADH, 50 mM 2-oxoglutarate.

3.4. Influence of ethanol on the response of the GLDH column.

Tolerance of immobilized GLDH *per se* to ethanol was evaluated by injecting 100 μl of 10 % ethanol solution more than 30 times into the FIA system. The activity of the GLDH column which was exposed to 10 % ethanol solution more than 30 times wasn't changed before the exposure. This result means that the immobilized GLDH is remarkably tolerant of ethanol.

Since the ammonia removal system would be used for the sample containing ethanol, the influence of ethanol on the response of the column was evaluated. The substrate solution (0.3 mM NADH, 10 mM 2-oxoglutarate, and 0.5 mM ammonium acetate) with or without 10 % ethanol was introduced into the system. No noticeable differences in the responses to the substrate solution with and without 10 % ethanol was exhibited.

3.5. Removal of ammonium ions by using GLDH column.

In order to investigate the removal ability of the immobilized GLDH column, the removal system (Fig. 1-(B)) was applied and 100 µl of substrate solutions with various concentrations of ammonium acetate was injected into the system. Since this system could measure the amount of ammonium ions, of which determination of the lower limit was 7.8 µM [23], the evaluation whether the removal of ammonium ion was done almost completely or not. As shown in Fig. 5, 100 µl of 0.5 mM ammonium acetate was removed completely when the solution include 0.6 mM NADH and 50 mM 2-oxoglutarate. The ability of removal of ammonium ion at the concentration of 0.5 mM was sufficiently applied to this determination system to for urea in rice wine because the concentration of endogenous ammonia molecules in the analyte of rice wine was below 0.2 mM.

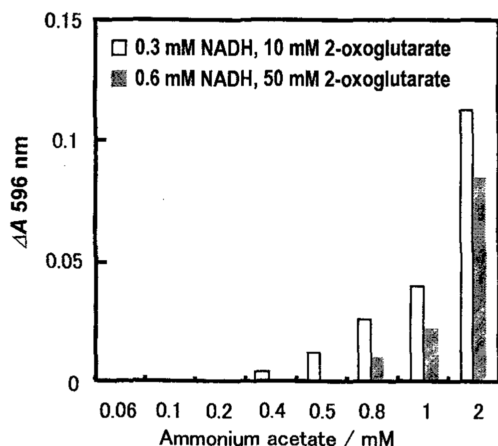


Fig. 5 Removal ability of ammonium ions by using the GLDH column.

4. Conclusion

In this study, we propose a useful removal system of ammonia by using the immobilized glutamate dehydrogenase column. The optimum pH for the GLDH column was to be 8.0, and the optimum constitution of the substrate solution for the ammonium determination was 0.6 mM NADH and 10 mM 2-oxoglutarate. The removal system of ammonia constructed in this study enabled ammonium ions to remove at the concentration of 0.5 mM. By using this system, endogenous ammonia could be successfully removed. The proposed system demonstrated that it should find use as a promising analytical tool for microdetermination of urea in alcoholic beverages. Furthermore, the system should be favorable for measuring urea in any samples without suffering from endogenous ammonia.

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