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Simple lysine sensing system using CO_2 electrode and enzyme immobilized to CNBr-activated sepharose 4B

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Abstract

A potentiometric L-lysine-selective sensor is described for the direct determination of lysine. The sensor system is based on a carbon dioxide gas sensing electrode and an L-lysine decarboxylase immobilized to CNBr-activated sepharose 4B. A highly selective L-lysine sensor has been prepared with immobilizing enzyme slurry put into reaction buffer solution. The optimum conditions for the measurement were evaluated by various experiments. This sensor exhibits a linear response to L-lysine concentrations from 10^{-4} M to 10^{-1} M. Response time of this lysine sensor is shorter than 30secs and the immobilized enzyme slurry is stable over one year.

Ⅰ. Introduction

The rapid, accurate, and selective assay of L-lysine is necessitated in several areas, especially food production industry. L-lysine is one of the essential amino acids and relates with the protein quality[1]. For this reason, it is very important to improve detection tool of the L-lysine content in food with a rapid simple method and immobilized enzymes have been used as a tool in food analysis[2]. These bioselective membrane electrodes based on an immobilized enzymatic system have been used for the selective and effective measurement of amino acids[3~6]. In this trend, Guilbault et al.[7] proposed an amino acid sensor using amino acid decarboxylase coated on CO_2 electrode. But the stability of the immobilized enzyme was very low. More recently, new fabrication technologies for CO_2 sensors have been developed using semiconductor processing[8] and quartz microbalance transducers[9].

To make a more developed and simple L-lysine detecting sensor system, we studied enzyme-substrate reaction and evaluated of CO_2 measurement by CO_2 gas-sensing electrode. The L-lysine sensor described in this paper could be useful to determine the L-lysine content in several kinds of foods.

Ⅱ. Principle

Principle of L-lysine sensor

L-lysine sensor is based on the detection of liberated CO_2 resulted from specific enzymatic decarboxylation of L-lysine by lysine decarboxylase as follows;

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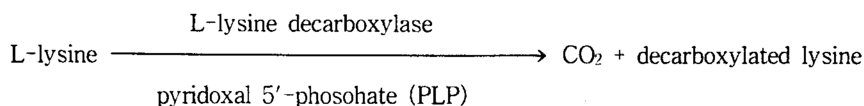

The carbon dioxide electrode uses a gas-permeable membrane to separate sample solution and electrode internal solution. Dissolved CO_2 in the sample which was derived from enzyme reaction diffuses through the gas-permeable membrane until an equilibrium state is reached between partial pressure of CO_2 in the sample solution and in the internal filling solution. As the amount of CO_2 derived from enzyme reaction changes, it affects the concentration of hydrogen ion in the internal filling solution. In any given sample the partial pressure of carbon dioxide will be proportional to the concentration of carbon dioxied ; $CO_2 + H_2O \longleftrightarrow H^+ + HCO_3^-$. So that we can measure the dissolved CO_2 concentration of sample solution through pH change using a pH electrode (inner body) located behind the membrane.

Ⅲ. Experimental

Apparatus

The used CO_2 electrode was Orion model (Cat. No. 95-02) CO_2 gas electrode and it was fitted on an apparatus shown in Figure 1 to make carbonate selective membrane electrode[4]. The electrode response was measured with millivolt meter (SYNTEX, sp-7) and recorded with Kipp & Zomen 912756 strip chart recorder using the cell shown in Figure 1. Most of measurements were carried out at room temperature with 2.2 mℓ reaction mixture. Samples were added to the reaction mixture in 5 mℓ beaker through an injection hole using Hamilton microsyringe. The enzyme reaction was initiated with magnetic stirring.

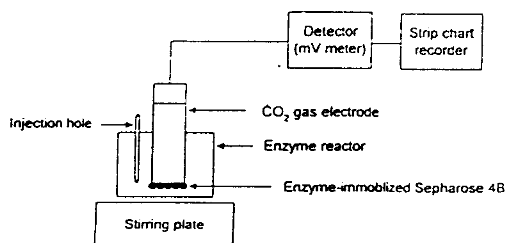

Fig. 1. Schematic diagram of lysine sensor system.

Reagents and materials

The L-lysine decarboxylase (1.6 mg solid, 21 units/mg solid, 121 units/mg protein), L-lysine, and pyridoxal 5′-phosphate were purchased from Sigma Chemical Co. (USA). CNBr-activated sepharose 4B was obtained from Pharmacia Chemical Co. (USA). Internal electrolyte for the CO_2 electrode was purchased from Orion Ionanalyzer (Cat. No. 95-02-02).

Coupling of lysine decarboxylase to CNBr-activated sepharose 4B

To couple enzyme to CNBr-activated sepharose 4B, lysine decarboxylase was dissolved in 2 mℓ of coupling buffer (0.10 M $NaHCO_3$, pH 8.3 containing 0.50 M NaCl). And 0.50 g of CNBr-activated sepharose 4B was swelled (gel formation) and washed with 1 ℓ of 1 mM HCl for 30 min. The coupling solution containing lysine decarboxylase was mixed with the gel in a stoppered vessel and rotated at 4℃ for overnight. The gel was transferred to glass filter and any remaining active groups were blocked by washing with an 0.10 M Tris-HCl buffer of pH 8.0 containing 0.50 M NaCl. Protein concentration of filtered solution was quantified by Bradford's method[10] and the coupling yield of enzyme was about 98 %.

Electrode measurement and enzyme reaction

Electrode measurements were performed by immersing the electrode in buffered sample and measured several times for each sample to confirm the reproducibility. Before injecting the sample to be measured, the electrode was immersed in buffer solution until to reach equilibrium state.

Typically, 2.0 $\mu\ell$ ~ 2.0 $m\ell$ of lysine samples were used for calibration according to its concentration. The electrode responses were noted as potential changes derived from the resulted pH variation as explained. Before the measurement, electrode was allowed to reach equilibrium state with 2.0 $m\ell$ of sodium acetate buffer, then L-lysine solution was injected for every measurement.

Ⅳ. Results and Discussion

Effect of different pH conditions of immobilized enzyme slurry

Amino acid decarboxylase was immobilized using CNBr-activated sepharose 4B and the enzyme slurry was tested for the effect of pH. After coupling of L-lysine decarboxylase to sepharose 4B, the immobilized enzyme slurry was stored in 0.10 M Tris-HCl buffer pH 8.0 containing 0.50 M NaCl at 4 ℃. Since the optimum pH of lysine decarboxylase has known to be pH 5.8[7], we examined the response of lysine sensor at different pH of immobilized decarboxylase. The first type of immobilized enzyme slurry was prepared by presoaking in 10^{-3} M sodium acetate (pH 5.8, 24 hrs). Storage condition to make the second type of immobilized enzyme slurry was 0.10 M Tris-HCl buffer, pH 8.0 containing 0.50 M NaCl. The last type of enzyme slurry was made by rinsing with 10^{-3} M sodium acetate (pH 5.8) by centrifugation (at 3000 rpm, 4℃, 5min) before enzyme reaction. Figure 2 shows the responses of CO_2 electrode using three types of each 200 $\mu\ell$ immobilized enzyme slurry

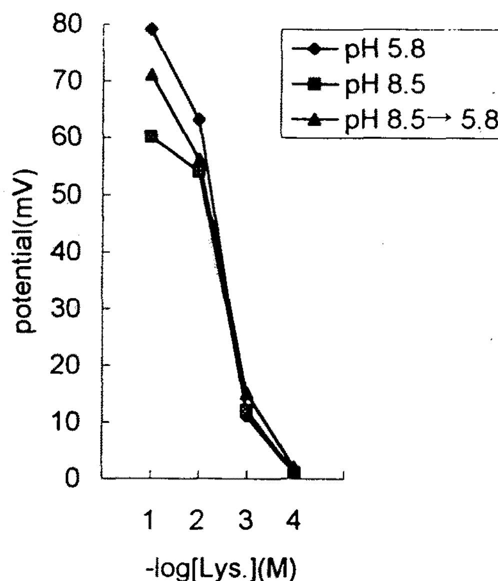

Fig. 2. Response of Lysine sensor at different pH of immobilized decarboxylase to sepharose.

(measuring condition: 10^{-3} M sodium acetate, pH 5.0, containing 10^{-3} M PLP). As a consequence, the third type of immobilized enzyme slurry was slightly more sensitive and stable than the other two. This is similar to the previously reported result[7]. Therefore, hereafter the third type of immobilized enzyme slurry was used for continous experiments in this study.

Effect of buffer concentration in reaction

To examine effect of sodium acetate concentration in reaction buffer, we tested the electrode response in a series of different concentration sodium acetate buffer - 10^{-4} M, 10^{-3} M, 10^{-2} M, 10^{-1} M - at a constant 10^{-3} M PLP and pH 5.0. Lysine samples are tested in each concentration of sodium acetate buffer containing 10^{-3} M PLP, pH 5.0 and 200$\mu\ell$ of immobilized enzyme to sepharose 4B. As shown in Figure 3, 10^{-3} M of sodium acetate buffer was relatively good so we use this condition for this study.

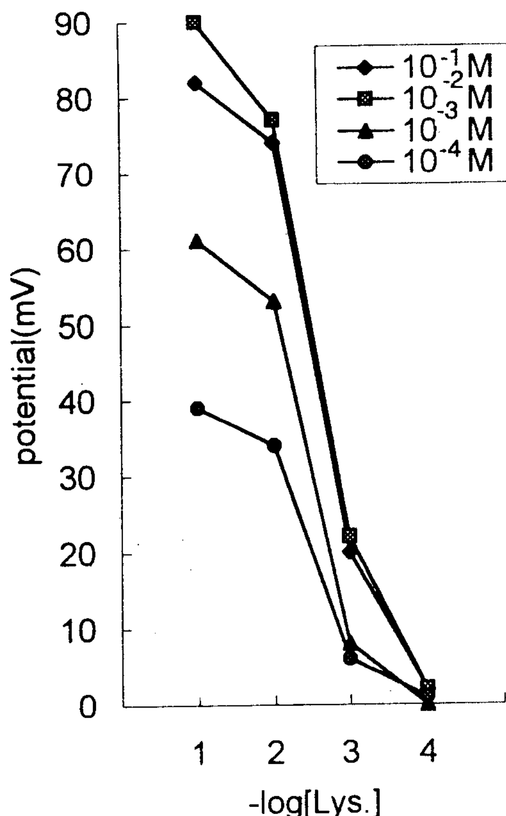

Fig. 3. Effect of sodium acetate concentration in reaction buffer on response of lysine sensor.

pH effect of reaction buffer

The pH at which this electrode exhibited maximum response was determined by testing the electrode response in a series of buffers at a constant concentration of sodium acetate and PLP (10^{-3} M sodium acetate, 10^{-3} M PLP). The acetate buffer was prepared by dissolving the sodium acetate in distilled water and adjusting the pH with acetic acid, sodium hydroxide, and hydrochloric acid. When various pH conditions (pH 3.9, pH 5.0, pH 5.8, and pH 7.4) were used, the response of lysine sensor was most sensitive at pH 5.0 as shown in Figure 4 which is very close to the optimum pH value reported for tyrosine sensor[3]. This includes the effect of pH on the enzyme reaction and also the CO_2 equilibrium. At pH 3.9 the response of lysine

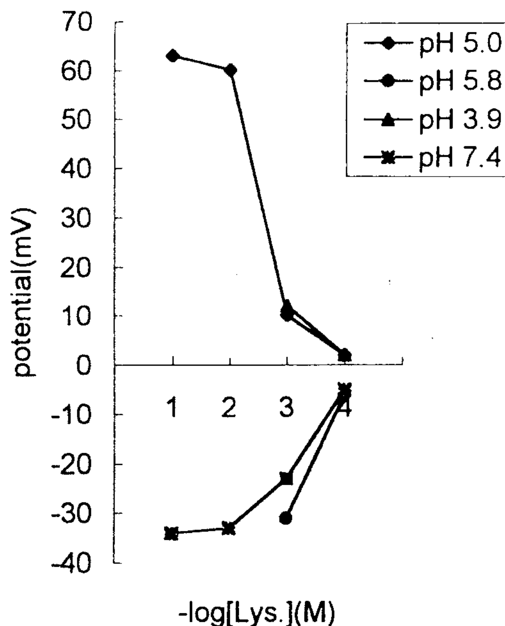

Fig. 4. Effect of pH in reaction buffer on responses of lysine sensor.

sensor is very low, and the potential response was shifted to negative direction at pH 5.8 and pH 7.4. Therefore pH 5.0 was selected as the optimum pH of lysine sensor operation. The pH optimum for this lysine sensor appears to be shifted slightly toward lower pH than the values given by White and Guilbault[7] which is pH 5.8.

Effect of PLP concentrations in reaction mixture

PLP (pyridoxal 5'-phosphate) is a cofactor for the reaction of L-lysine and lysine decarboxylase. A series of reaction buffer was prepared at a constant concentration of 10^{-3} M sodium acetate, pH 5.0, but each buffer contained 10^{-4} M, 10^{-3} M, and 10^{-2} M PLP, respectively. Regardless of PLP concentrations in sodium acetate buffer the response of lysine sensor was very satisfactory as shown in Figure 5. From these experiments, we selected 10^{-3} M sodium acetate buffer, pH 5.0 containing 10^{-3} M PLP as optimum conditions of reaction buffer in this lysine sensor.

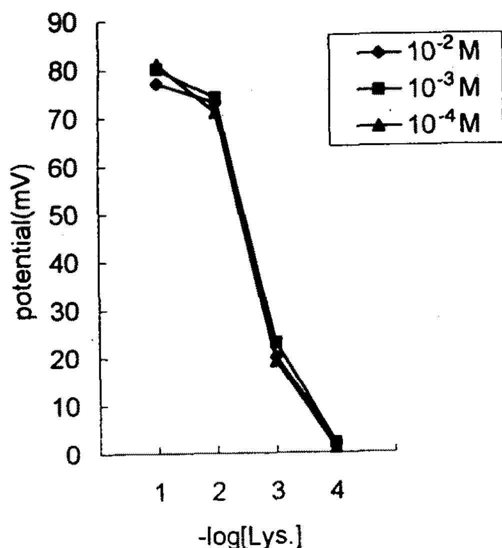

Fig. 5. Effect of PLP concentration in reaction buffer on response of lysine sensor.

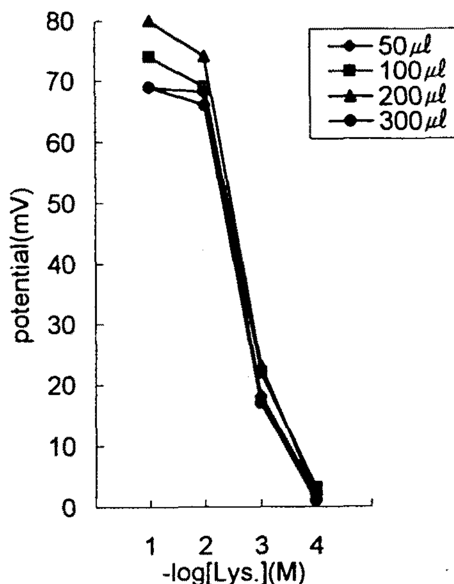

Fig. 6. Effect of enzyme slurry volume in reaction buffer on responses of lysine sensor.

Quantity of immobilized enzyme slurry in reaction mixture

The effect of enzyme amount on electrode response was investigated. The amount of immobilized enzyme slurry was varied as 50 µℓ, 100 µℓ, 200 µℓ, and 300 µℓ, respectively, while other conditions of reaction buffer remained essentially the same. The results are given in Figure 6 and the highest sensitivity was shown when 200 µℓ enzyme slurry was used in reaction buffer. Lysine sensor showed that electrode responses of 50 µℓ slurry and 300 µℓ slurry were lower than that of 100 µℓ or 200 µℓ slurry. When the amount of enzyme slurry was 100 µℓ and 200 µℓ, there was little difference on electrode response for 10^{-4} M to 10^{-2} M lysine. But in the 10^{-1} M lysine, the response of 200 µℓ slurry was 6 mV higher than that of 100 µℓ slurry. These results may be interpreted by a diffusion velocity into the reactive enzyme layer of electrode. But, unfortunately, we couldn't explain the relationship between enzyme amount and electrode response precisely and now under studying.

Effect of enzyme reaction temperature

Figure 7 illustrates the response of lysine sensor where the enzyme reaction temperature was varied to 4 ℃, 25 ℃, 37 ℃, respectively, and other conditions were controlled to optimum condition established by previous experiments as mentioned above. The response at 4℃ was lower and also response time was longer than at the other two temperatures, at 25 ℃, and 37 ℃. The response time was shorter than 30 secs and the sensitivity of lysine sensor was improved at 25 ℃ or 37 ℃. Therefore, lysine sensor could be useful to measure at room temperature effectively. Other amino acid sensor developed by potentiometric method was more sensitive at 37 ℃ than room temperature[3].

Response of lysine sensor by $NaHCO_3$ and lysine.

From the previous experiments, we established the appropriate conditions on response of lysine

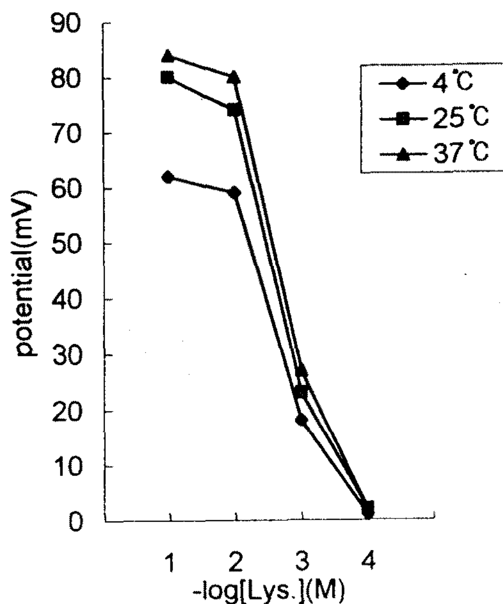

Fig. 7. Effect of enzyme reaction temperature on performance of lysine sensor.

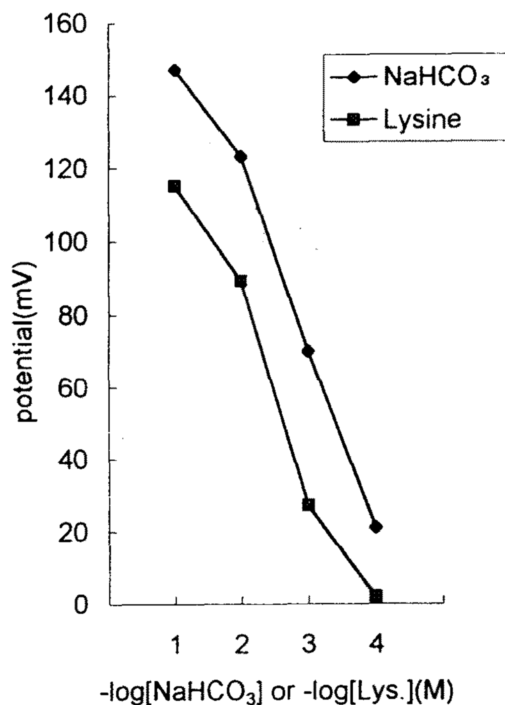

Fig. 8. Responses of lysine sensor with various concentrations of $NaHCO_3$ and lysine in optimum conditions established from Fig. 2~Fig. 7.

sensor. Figure 8 confirms a similar and reasonable tendency through the comparing of lysine sensor responses with those of $NaHCO_3$ standard solution in the established condition ($NaHCO_3$ can liberate CO_2 gas directly without enzyme reaction). The response of this sensor was slightly higher with $NaHCO_3$ sample than lysine sample. This could be due to the interferents such as decarboxylated lysine which is the product of the enzyme reaction rather than the volatility of CO_2 itself.

By the resulted sensor system and measuring condition, detection of lysine could be performed in the 10^{-1} M ~ 10^{-4} M lysine concentration. So to measure the higher lysine concentration than 10^{-1} M, samples should be diluted before measuring to obtain accurate response.

Recycling of enzyme-immobilized slurry and its long term stability.

The enzyme activity of immobilized enzyme slurry was stable in 0.10 M Tris-HCl buffer, pH 8 containing 0.50 M NaCl at 4 ℃ over 1 year during storage. Used enzyme slurry after its enzyme reaction was collected and rinsed by centrifugation. This recycled immobilized enzyme slurry exhibited the same activity of newly maded enzyme and could reuse over 30 times. The reproducibility of response or precision associated with this electrode was also quite good. When four to six replicate samples are run at several concentrations for L-lysine, the average standard deviation obtained is ± 4-5 mV or less. Physically entrapped enzymes present generally lower stabilities than chemically immobilized enzymes. Under ideal conditions a physically entrapped enzyme may be stable for 3 weeks or 100 assays and a chemically bound enzyme for over a year and up to 1000 assays[11]. Accordingly, mentioned enzyme immobilization method is an economical way for this lysine sensor system.

In conclusion, the lysine sensor system with

CO_2 gas electrode and L-lysine immobilized decarboxylase beads using CNBr-activated sepharose 4B has been developed to improve the reliability and stability of the lysine measurement in the solution sample. Although, accurate explanations of all the examined results were impossible and necessitate more studies, its response time and the stability of enzyme based on the derived condition from this study were sufficient to measure lysine concentration continuously.

In addition, this sensor system is very simple and recycling of used immobilized-enzyme is very convenient to reuse and the reliability of the system was proved by comparing electrode responses of $NaHCO_3$ with L-lysine standard solution. So that this measuring system could be useful in food and nutrition fields and also applied for the other amino acid sensors using amino acid decarboxylase system.

References

[1] J. L. Groff, S. S. Gropper, and S. M. Hunt, Advanced Nutrition and Human Metabolism, 2nd Ed., West Pub. Co., 154-190, 1995.

[2] G. Schwedt and K. Stein, "Immobilized enzymes as tools in food analysis", Z. Lebensm. Unters. Forsch., Vol. 199, 171-182, 1994.

[3] J. Havas and G. Guilbault, "Tyrosine-selective enzyme probe and its application", Anal. Chem., Vol. 54, 1991-1997, 1982.

[4] P. M. Kovach and M. E. Meyerhoff, "Development and application of a histidine-selective biomembrane electrode", Anal. Chem., Vol. 54, 217-220, 1982.

[5] P. Valle-Vega, C. T. Young, and H. E. Swaisgood, "Arginase-urenase electrode for determination of arginase and peanut maturity", J. Food Science, Vol. 45, 1026-1030, 1980.

[6] K. Mstsumoto, O. Hamada, H. Ukeda, and Y. Osajima, "Amperometric flow injection determination of fructose with an immobilized fructose 5-dehydrogenase reactor", Anal. Chem., Vol. 58, 2732-2734, 1986.

[7] W. C. White and G. C. Guilbault, "Lysine specific enzyme electrode for determination of lysine in grains and foodstuffs", Anal. Chem., Vol. 50, 1481-1486, 1972.

[8] T. Lang, M. Caran, R. Izquierdo, D. Ivanov, J. F. Currie, and A. Yelon, "Material characterization of sputtered sodium-ion conductive ceramics for a prototype CO_2 micro-sensor", Sensors & Actuators B, Vol. 31, 9-12, 1996.

[9] R. Zhou, D. Schmeisser, and W. Gopel, "Mass sensitive detection of carbon dioxide by amino group-functionalized polymers", Sensors & Actuators B, Vol. 33, 188-193, 1996.

[10] M. M. Bradford, "A rapid and sensitive method for the quantization of microgram quantities protein utilizing the principle of protein dye binding", Anal. Biochem., Vol. 72, 248-256, 1976.

[11] G. G. Guilbault, "Analytical uses of immobilized enzymes", Marcel Dekker, New York, 1984.

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