

Glucose 정량을 위한 *Citrobacter freundii* Bio-Sensor의 개발과 그 응용

印權植 · 洪永錫 · 金宣洛 · 張世容 · 孫武正[†]

계명대학교 자연과학대학 화학과

(1989. 8. 22 접수)

Preparation of the *Citrobacter freundii* Bio-Sensor for the Determination of Glucose and Its Applications

Gwon-Shik Ihn, Young-Seuk Hong, Ui-Rak Kim, Seh-Yong Jang, and Moo-Jeong Sohn[†]

Department of Chemistry, College of Natural Science, Keimyung University, Taegu 704-200, Korea

(Received August 22, 1989)

요 약. *Citrobacter freundii*와 그의 organelle을 CO₂ gas-sensor에 고정시켜서 glucose 정량을 위한 bio-sensor를 조립하였다. 박테리아의 경우가 organelle 보다 감응은 우수하였으나 감응시간은 organelle의 경우가 훨씬 짧았다. 박테리아 전극은 30°C의 온도에서 pH 7.0인 0.2 M tris-HCl 완충용액을 사용하면 $7.0 \times 10^{-4} - 1.0 \times 10^{-2}$ M glucose 농도범위에서 42.2 mV/decade의 감응기울기를 나타냈다. 이 전극은 glucose에 대해서 선택성이 매우 우수하였으며, 혈청속의 glucose 정량에 사용한 결과 routine analyzer와 좋은 일치를 보였다.

ABSTRACT. A bio-sensor for the determination of glucose has been constructed by immobilizing the *Citrobacter freundii* or its organelle on carbon dioxide gas-sensor. The bacterial sensor was better than organelle in response, but the latter showed a shorter response time. The bacterial sensor gave linearity between 7.0×10^{-4} and 1.0×10^{-2} M glucose with a slope of 42.2 mV/decade in pH 7.0, 0.2 M tris-HCl buffer at 30°C. The selectivity of this sensor was very high for glucose. Employing for the determination of glucose in serum, the sensor showed a good agreement with a routine analyzer.

INTRODUCTION

The accurate determination of glucose for the diagnosis and treatment of diabetes is an important test carried routinely out in clinical laboratories. The methods of glucose determination can be classified into three groups: chemical, enzymatic and electrochemical.

Most chemical measurement of glucose depends upon its reducing properties, and because of the lack of specificity most are no longer used. However o-toluidine is the only chemical method still used widely, but its major disadvantage is the

corrosiveness of the reagent to laboratory equipment as well as its toxicity¹.

Enzymatic methods have been developed in order to increase the specificity of glucose estimations. Glucose can be measured by its reaction with glucose oxidase, in which gluconic acid and hydrogen peroxide are formed. The hydrogen peroxide then reacts with chromogenic oxygen acceptors, such as scopoletin², o-dianisidine³, o-toluidine⁴ and homovanillic acid⁵, in the reaction catalyzed by peroxidase to form a color.

In this method, one of the disadvantages is that numerous reducing substances inhibit oxidation of

chromogens used in the peroxidase-reaction.

Recently many electrochemical methods utilizing glucose oxidase have been developed for the determination of glucose ⁶⁻¹¹. The most noteworthy of these is the bio-sensor that is constructed by immobilizing glucose oxidase on an oxygen electrode. In this method, the reaction of glucose with oxygen is monitored by an oxygen-sensing electrode. By determining the amounts of oxygen consumption, glucose can be estimated. But in the bio-sensor using enzyme (enzyme electrode), they are not suitable for the determination of glucose in biological fluids because enzyme are generally expensive and unstable than living cells ¹².

This paper describes a bacterial sensor method for the determination of glucose by direct potentiometric measurement of the carbon dioxide produced in the processes of oxidative phosphorylation of TCA (citric acid) cycle. The bacterial sensor is constructed by placing a immobilized layer of *Citrobacter freundii* on a carbon dioxide gas-sensor. And the organelle sensor is constructed by immobilizing the ribosomes extracted from the microorganisms on a carbon dioxide gas-sensor, which is compared with the bacterial sensor in terms of response slope, linear range and response time. Finally, the bacterial sensor method was directly compared with the routine nanalyzer using widely in clinical laboratories.

EXPERIMENTAL

Apparatus. An Orion 95-02 carbon dioxide gas-sensor was employed in the construction of the bio-sensor for glucose. Potentials were measured with an Orion Model 901 Digital microprocessor Ionalyzer. The pH of the solutions was measured with a Beckman Model 76 Century SS with a combination glass electrode. The temperature of samples was controlled by Forma Scientific Bath and Circulator-2067. Hitachi 70P-72 ultra-centrifuge

was employed for the collection of cells and organelle. A Beckman ASTRA-8 Routine Analyzer was used for the determination of glucose in serum.

Materials. *Citrobacter freundii* was obtained from the Department of Microbiology, School of Medicine, Keimyung University, and identified as previous work ¹³. The microorganisms were grown on Nutrient agar (Difco Lab.) plate for the construction of bacterial sensor ¹⁴ and in Nutrient broth (Difco Lab.) medium ¹⁵ for the extraction of organelle. Stock solutions of glucose were prepared with β -D(+) glucose (Sigma Chemical Co.) in buffer solutions, which were prepared from NaH_2PO_4 and Na_2HPO_4 , and tris (hydroxymethyl) aminomethane (Sigma Chemical Co.) and HCl. Other reagents, except as noted, were commercially available reagents or laboratory grade materials.

Deionized water used in all procedures.

Extraction of Organelle. About 100g of frozen cells obtained from Nutrient broth medium (liquid cultivation) was disrupted at 2,000 psi on a Carver Laboratory C-29000 Press for 2 hour as shown in Fig. 1. The assembly was stored at -20°C for 3 hours before use. DNase (Sigma Chemical Co.) 0.5 mg was added to the buffer (10 mM tris-HCl, pH 7.5; containing 1 mM dithiothreitol (DTT) and 10 mM MgCl_2) containing disrupted cells, and whole cells and cell debris were removed by centrifugation at 30,000g for 30 min. Ribosomes were sedimented when the supernatant solution was clarified by centrifugation at 150,000 for 3 hours. All procedures to extract ribosomes were carried out at 4°C .

Preparation of Glucose Sensor. The cultured microorganisms or the sedimented ribosomes were then applied as a paste to the dialysis membrane (cellulose nitrate, pore size $0.22\mu\text{m}$, Millipores Co.) and held in place by means of a gas-permeable membrane. The sensor was conditioned in 0.2 M tris-HCl buffer solution for several hours before use. This prepared sensor was stored in the same

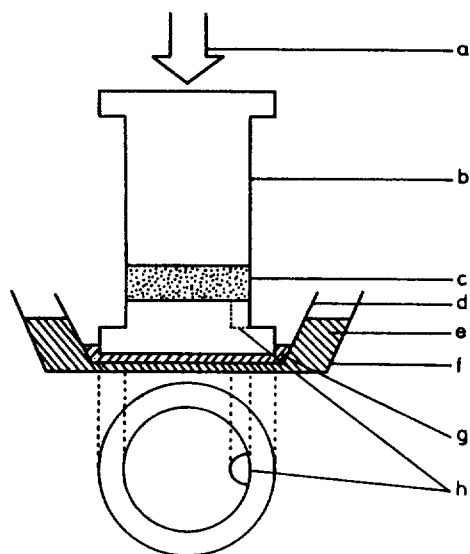


Fig. 1. Schematic diagram for the homogenization of cells: a. pressing; b. stainless steel; c. frozen cells; d. aluminum foil; e. 10 mM tris-HCl buffer containing 1 mM DTT and 10 mM MgCl_2 ; f. stainless bowl; g. disrupted cells; h. hole.

buffer at 4°C when not in use.

RESULTS AND DISCUSSION

Activity of the Microorganism. Fig. 2 shows the dependence of the activity of the microorganism on cultivation times in the range of 10^{-2} – 10^{-3} M glucose solutions. When the microorganisms were cultured for over 24 hours, the response slope reached maximum, 42.2 mV/decade. Thus the microorganisms were employed after culture for 24 hours.

Calibration curve. Fig. 3 is a typical calibration curve for the bacterial glucose sensor in pH 7.0, 0.2 M tris-HCl buffer. The sensor has the linearity within the concentration range of 7.0×10^{-4} – 1.0×10^{-2} M glucose with a slope of 42.2 mV/decade at 30°C .

It is expected that this linear ranges is suitable for the determination of physiological glucose, because glucose in the serum of the average adults

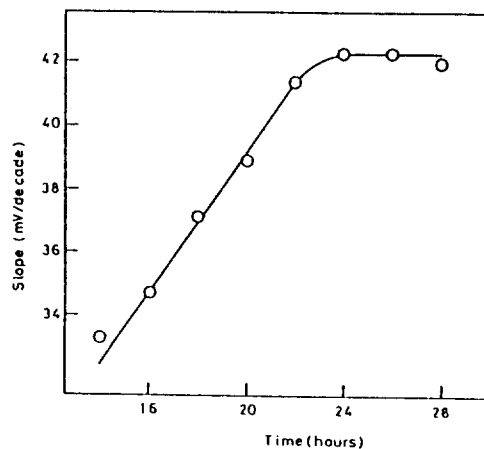


Fig. 2. Activation curve of microorganisms *Citrobacter freundii*.

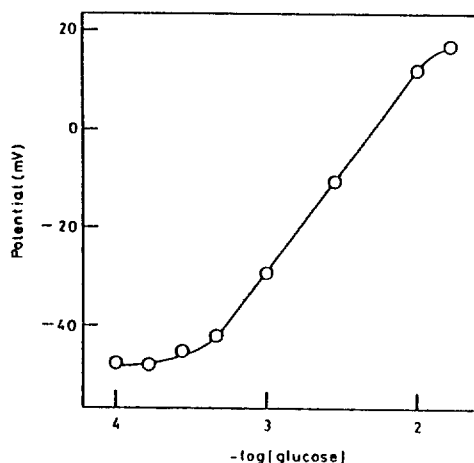


Fig. 3. Calibration curve for glucose in 0.2 M tris-HCl buffer pH 7.0 at 30°C .

is approximately 5.0×10^{-3} M.

Effect of pH and temperature. The influence of pH on the response of the sensor was examined at various pH values in 0.2 M tris-HCl buffer. The response to glucose was changed little between pH 6.8 and 7.2 as shown in Fig. 4. The linearity appeared in the range of 7.0×10^{-4} – 1.0×10^{-2} M glucose at pH 6.8 and 7.0, 9.0×10^{-4} – 1.0×10^{-2} M at pH 7.2.

Hence pH 7.0 was used for all further works because of the wider linear range, although relatively a good response (42.9 mV/decade) was obtained

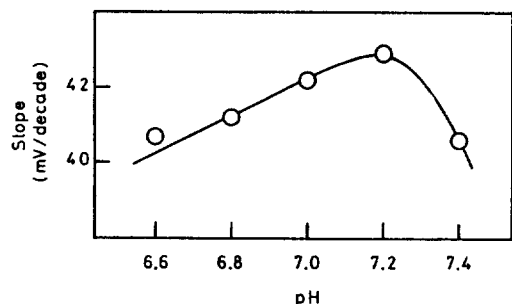


Fig. 4. Effect of pH on the glucose bacterial sensor in tris-HCl buffer at 30 °C.

Table 1. The effect of buffer solutions on the bacterial sensor for glucose.

Buffers	Slope (mV/decade)	Linear range (M)
0.05 M Tris-HCl	36.0	$1.0 \times 10^{-3} - 1.0 \times 10^{-2}$
0.1 M Tris-HCl	39.0	$9.0 \times 10^{-4} - 1.0 \times 10^{-2}$
0.2 M Tris-HCl	42.2	$7.0 \times 10^{-4} - 1.0 \times 10^{-2}$
0.05 M Phosphate	30.2	$1.0 \times 10^{-3} - 3.0 \times 10^{-2}$
0.1 M Phosphate	35.9	$9.0 \times 10^{-4} - 1.0 \times 10^{-2}$
0.2 M Phosphate	32.0	$1.0 \times 10^{-3} - 3.0 \times 10^{-2}$

at pH 7.2.

The effect of temperature on this sensor was examined in the range of 25–35 °C. The sensor showed the best response at 30 °C, which has a slope of 42.2 mV/decade in the range of $7.0 \times 10^{-4} - 1.0 \times 10^{-2}$ M glucose, and thus temperature for this sensor was set at 30 °C for all experiments.

Effect of Buffer. The effect of buffer solution on the response to glucose is shown in Table 1. This sensor was very sensitive in tris-HCl buffer rather than in phosphate, and 0.2 M tris-HCl buffer showed the best responsibility and the widest linearity. Thus all experiments were carried out in 0.2 M tris-HCl buffer.

Table 2. The effect of interference on the bacterial sensor for glucose.

Substances ^a	Response (mV)	ΔmV
Fructose	-36.4	2.6
Sucrose	-39.0	0
Rhamnose	-39.0	0
Mannose	-39.0	0
Arabinose	-38.6	0.4
Raffinose	-38.5	0.5
Maltose	-39.0	0
None ^b	-39.0	0

^aAdded 1.0×10^{-3} M substances to equimolar glucose.

^bPure 1.0×10^{-3} M glucose.

Table 3. The effect of inorganic salts on the bacterial sensor for glucose.

Substances ^a	Response (mV)	ΔmV
FeCl ₃ ·6H ₂ O	-39.0	0
CuCl ₂ ·2H ₂ O	-43.4	4.4
MnCl ₂ ·4H ₂ O	-39.6	0.6
MgCl ₂ ·6H ₂ O	-40.1	1.1
NaCl	-39.0	0
KCl	-39.0	0
KNO ₃	-39.0	0
Na ₂ SO ₄	-39.0	0
NaAc	-40.3	1.3
NaNO ₃	-39.0	0
None ^b	-39.0	0

^{a,b}were the same as described in Table 2.

Interferences. Table 2 and 3 show the effects of various saccharides and inorganic salts on this sensor for glucose, respectively. The degree of interferences, ΔmV , displays the differences in the responses of them and pure glucose. As shown in Table 2, only fructose was found to be small interference. Therefore the selectivity of this sensor is very high for glucose.

On the other hand, it appears that most inorganic anions do not have an effect on the response of the sensor but several metal cations, such as copper, manganese and magnesium, reacted as an inhibitor, as shown in Table 3. Especially, it is a very good thing for the determination of glucose in se-

Table 4. Comparison of bacterial and organelle sensor in responsibility for glucose.

Sensors	Slope (mV/decade)	Linear range (M)	Response time (min)
Bacterial	42.2	7.0×10^{-4} 1.0×10^{-2}	15-20
Organelle	14.3	7.0×10^{-4} 3.0×10^{-2}	5-10

rum that potassium and sodium ions, which are abundantly contained in physiological fluids, not given any interferences.

Response time and Stability. This bacterial glucose sensor had a response time of 15-20 min in optimum conditions, pH 7.0, 0.2 M tris-HCl buffer and 30°C. The response time grew longer little by little with elapsed time, that is, 17-21 min was required in the 5th day; 19-23 min, 20-24 min were required in the 7th day 10th day, respectively.

Whereas the sensor changed little in the response slope and linearity until the 5th day. However, the sensor also gave good reproducibility in the response and linearity after then.

Organelle Sensor. Table 4 shows the comparison of bacterial and organelle glucose sensors. The bacterial sensor is better than the organelle in response, but the latter required a shorter response time, 5-10 min than the former.

On the other hand, when the microorganisms were homogenized by ultrasonic, mortar and pestle, and glass bead beater, the organelle never responded to glucose. Judging from these results, it is presumed that the enzyme in the organelle was greatly damaged by physical force.

Hence, if new extraction methods for the organelle are developed, better results may be obtained in its response to glucose.

Clinical applications. This bacterial sensor prepared in this study was employed for the determination of glucose in serum. Whole bloods were quickly clarified by centrifugation in order to avoid the glycolysis after drawing bloods.

Table 5. Comparison of routine analyzer and bio-sensor in clinical application for serum glucose.

Sample No.	Routine ^a analyzer	Bio ^a sensor	Difference (%)
Kang	91	92	1.10
Jang	101	98	2.97
Sohn	109	107	1.87

^aUnit: mg/dl.

Then sera were diluted 3 times and their glucose concentrations were determined on the calibration curve. As shown in Table 5, the results showed good agreement with a routine analyzer within $\pm 3\%$ difference.

ACKNOWLEDGEMENT

We are grateful to the Ministry of Education for the financial support of this work, and Dr. I.Y. Kang and Mr. W.H. Lee of Youngnam University hospital for the routine analytical help.

REFERENCES

1. A. Indrikson, *Clin. Chem.*, **21**, 1345 (1975).
2. R. Bonnichsen and C. Lundgren, *Acta Pharmacol. Toxicol.*, **13**, 256 (1957).
3. H. V. Malmstadt and G. P. Hicks, *Anal. Chem.*, **32**, 394 (1960).
4. W. J. Blaedel and G. P. Hicks, *Anal. Chem.*, **34**, 388 (1962).
5. G. G. Guilbault, P. Bringnac, and M. Zimmer, *Anal. Chem.*, **40**, 190 (1968).
6. G. Nagy, L. H. von Storp, and G. G. Guilbault, *Anal. Chim. Acta*, **66**, 443 (1973).
7. M. Aizawa and S. Suzuki, *Denki Kagaku (Japan)*, **44**, 279 (1976).
8. D. R. Trevenot, R. Strenberg, P. R. Coulet, J. Laurent, and D. C. Gautheron, *Anal. Chem.*, **51**, 96 (1979).
9. M. Koyama, Y. Sato, M. Aizawa, and S. Suzuki, *Anal. Chim. Acta*, **116**, 307 (1980).
10. A. E. G. Cass, C. Davis, G. D. Francis, H. A. O. Hill, W. J. Aston, I. J. Higgins, E. V. Plotkin, L. D. L.

- Scott, and A. P. F. Turner, *Anal. Chem.*, **56**, 667 (1984).
11. M. Mascini and A. Memoli, *Anal. Chim. Acta*, **182**, 113 (1986).
12. S. Bascomb and C. A. Grantham, in R. G. Broad, and D. W. Lovelock (Eds.), *Some Methods for Microbiological Assay*, Academic Press, New York, 1975, p. 29.
13. G. S. Ihn and B. W. Kim, *J. Kor. Chem. Soc.*, **32**(4), 333 (1988).
14. G. S. Ihn, S. T. Woo, M. J. Sohn, and R. P. Buck, *Anal. Lett.*, **22**(1), 1 (1989).
15. G. S. Ihn, B. W. Kim, M. J. Sohn, and I. T. Kim, *J. Kor. Chem. Soc.*, **32**(4), 323 (1988).