Paper

Amperometric Glucose Sensor Using Thermostable Co-Factor Binding Glucose Dehydrogenase

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A thermostable mediator-type enzyme glucose sensor was constructed. The electrode was fabricated using chemically cross-linked thermostable co-factor binding glucose dehydrogenase (GDH) from thermophilic bacteria in carbon paste matrix. The electrode responded directly proportional to D-glucose concentration from 0.01 mM to 3 mM in stirred buffer containing 1 mM 1-methoxyphenazinemethosulfate as a mediator with the steady-state mode. The storage stability was examined by incubating the enzyme electrode at 50°C during the measurement. The cross-linked GDH immobilized electrode showed good storage stability. Ninety percent of its initial response was retained after incubation in buffer solution for 9 days at 50°C. The flow injection analysis (FIA) glucose sensing system was also constructed by immobilizing the cross-linked GDH and ferrocene as a mediator in the carbon paste matrix. The FIA system was able to measure 600 samples for 100 h.

Keywords : glucose dehydrogenase, thermostability, cross-link, co-factor binding, glucose sensor, FIA system

1. Introduction

Glucose oxidoreductases that are the enzymes that catalyze glucose oxidation have been widely utilized in industry, particularly as a component for the various enzyme sensors in the measurement of fermentation broth and the clinical diagnosis of diabetes. Various glucose oxidoreductases have been reported and they can be divided into two groups based on their electron acceptors: 1) glucose oxygen-oxidoreductases (GOD) (EC1.1.3.4.) and 2) glucose dehydrogenases (GDH) such as glucose 1-dehydrogenase (EC1.1.1.47.) and dye mediated glucose dehydrogenases such as pyrroquinoline quinone (PQQ) dependent GDH (EC1.1.99.17.).

The glucose sensors utilizing glucose oxidase (GOD) have been well studied and various types of glucose sensors were commercialized. Recent trends in glucose sensor for blood glucose monitoring are the electrochemical sensors employing electron mediator, which can be operated at ca. 100 mV vs Ag/AgCl, at which potential the existence of various compounds to be oxidized in blood are negligible. However, due to the inherent problem of GOD, that GOD utilizes oxygen as the electron acceptor, the application of GDH for mediator type glucose sensors is recently being focused.

PQQ dependent GDH, which is an electron mediator-dependent glucose dehydrogenase, is higly focused as an ideal molecular recognition device of glucose sensor because of its negligible effect of the presence of oxygen in the reaction mixture [(1)~(9)]. This PQQ dependent has high catalytic activity [(3)], however, the stability of the enzyme was somewhat inferior to GOD. It is necessary to obtain the thermostable enzyme.

Among various GDHs, some are isolated from thermophilic bacteria[(10) ~ (12)], however, these thermostable GDHs are all NAD(P)-dependent GDHs, which require continuous additions of cofactor, NAD(P), to show electron-mediator-dependent GDH activity. There is a problem of enzyme sensor using NAD(P)-dependent enzymes, because cofactor, NAD(P) should be transported from the enzyme active site to the electrode but fixation of the coenzyme on the sensor surface limits the transport. For this reason, NAD(P)-dependent GDHs are not suitable for glucose sensoring element. The cofactor-binding thermostable GDHs are required for glucose sensor but have never been reported.

In order to obtain a thermostable enzyme for glucose sensor construction, we have been carrying out the screening of glucose dehydrogenase from thermophilic bacteria[(13)]. As a result, we obtained a GDH from a moderate thermophile strain SM4. This GDH is co-factor-binding type and does not require additional co-factors[(13), (14)]. This GDH consists of two distinct peptides; an α-subunit, molecular weight is 67 kDa, and ß-subunit, 43 kDa. Thermal denaturation of this GDH caused dissociation of subunits. In order to avoid thermal dissociation of this GDH, we chemically cross-linked these subunits with 1% of glutaraldehyde[(15)]. As a result, the thermal dissociation was avoided without decrease of its initial activity, consequently the GDH retained more than 90% of initial activity even after 30 min incubation at 65°C. Our GDH is the most stable GDH so far reported, therefore, the application of this enzyme for the sensor construction will enable us to construct a practical glucose sensor system employing GDH.

The aim of this work is to construct a stable glucose sensor employing cross-linked GDH from the strain SM4. We first

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constructed the enzyme electrode utilizing thermostable cross-linked GDH from the strain SM4, and characterized its storage stability by batch experiments. Then we also constructed the flow injection analysis (FIA) glucose sensing system with the enzyme electrode utilizing cross-linked GDH, and studied the operational stability of the system.

2. Experimental

2.1 Chemicals Glucose was purchased from Kanto Chemicals (Tokyo, Japan). Glutaraldehyde was purchased from Kishida Chemicals (Tokyo, Japan), 1-methoxyphenazine methosulfate (m-PMS) and ferrocene were from Wako Chemicals (Osaka, Japan), carbon paste from BAS Inc. (Indiana, USA), and mineral oil from SIGMA (Missouri, USA). The other reagents were of analytical grade.

2.2 Enzyme Preparation A bacterial strain producing a thermostable GDH, SM4, was cultured as described previously [13]. Cells were resuspended in 10 mM potassium phosphate buffer (pH 6.0), and disrupted by French pressure cells (Ohtake seisakusyo, Tokyo, Japan) at a pressure difference of 1,500 kg cm^{-2}. The cell extract was centrifuged at 9,000 xg for 20 min to remove cell debris. The supernatant fluid was ultracentrifuged at 40,000 xg for 90 min at 4°C. The membrane fraction was collected as pellets. From 30 g of wet cells, approximately 2 g of membrane fraction were collected.

2.3 Preparation of the Enzyme Electrode The cross-linked GDH was prepared as follows. One milligram of the membrane fraction containing GDH was suspended in 10 ml of 10 mM potassium phosphate buffer (pH 6.0), followed by addition of 1% of glutaraldehyde solution, and stirred for 30 min at room temperature.

The cross-linked GDH or the intact GDH (the intact GDH was the membrane fraction suspended in 10 mM potassium phosphate buffer, pH 6.0, and not treated with glutaraldehyde solution) was added to carbon paste and lyophilized. For batch type sensor, 2.5 units of the cross-linked GDH was added to 50 mg of carbon paste, and 3 units of that was added to 60 mg of carbon paste for FIA system. For FIA system, 10 mg ferrocene was also mixed into the carbon paste containing enzyme. Then mineral oil was added to the paste and mixed in (40 μL of mineral oil was added per 50 mg of carbon paste). The resulting paste was filled into the body of the carbon paste electrode (inner diameter 3 mm, geometric surface area of 0.28 cm^2, BAS Inc.). Its surface was gently rubbed with filter paper to produce a flat shining electrode surface. The enzyme electrodes were stored in 0.1 M potassium phosphate buffer (pH 7.0), at 4°C unless stated elsewhere.

2.4 Electrochemical Measurement For steady-state mode, an Ag/AgCl (Model RE-1, BAS Inc.) and Pt wire were used as reference and counter electrodes, respectively. The enzyme electrode (3 mm diameter, BAS Inc.), reference electrode, and counter electrode were located in the 10 mL water-jacket cell (BAS Inc. Model VC-2) through holes in its Teflon cover. The potential was controlled by a potentiostat (HOKUTO-DENKO, Tokyo, Japan) in a three-electrode cell and currents were recorded with a recorder (Ohkura electric company, Tokyo, Japan). The applied potential was +0.25 V vs. Ag/AgCl. The steady-state cell was always under stirred conditions (250 rpm) with a magnetic stirrer at 25°C.

For FIA system, the enzyme electrode was mounted in a flow through amperometric cell (BAS Inc., Model No.11-2456) as the working electrode. An Ag/AgCl and stainless steel tube served as reference and counter electrodes, respectively. The carrier solution was 0.1 M potassium phosphate buffer (pH 7.0) and the flow rate was 0.5 mL min^{-1}. Glucose samples (20 μL) were injected using an autosampler (TOSOH, Japan). The potential of the enzyme electrode was set at +0.40 V vs. Ag/AgCl. The measurement was performed at room temperature.

3. Results and Discussion

3.1 Electrochemical Rsponse of the Ezyme Electrode Immobilizing GDH The GDH immobilized electrode was immersed in a 0.1 M potassium phosphate buffer (pH 7.0), containing 1 mM m-PMS. Cyclic voltammograms were investigated at potential ranges between - 0.3 V and + 0.3 V vs. Ag/AgCl with a scan rate of 20 mV s^{-1}. Peaks of cyclic voltammogram due to the electrochemical redox reaction of m-PMS on the surface of the electrode were observed and it was increased according to the addition of glucose. The anodic applied potential was set at + 0.25 V. Figure 1 shows the typical response curve of the cross-linked GDH immobilized electrode in the steady-state experiments. One hundred microliter of 100 mM glucose was successively added into 10 mL of the basic solution. With the addition of glucose solutions, the anodic current reached its steady state within 3 min. Figure 2 shows the relationships between the anodic current and the final concentration of glucose. The cross-linked GDH immobilized electrode showed linear response in the range of 0.01 mM to 3 mM final glucose concentration.

The intact GDH immobilized electrode also responded to glucose addition, however, the response was one-twentieth of the response to that of the cross-linked GDH immobilized one. We previously reported that the cross-linking procedure stabilized the GDH structure[15] and this result shows that the cross-linking

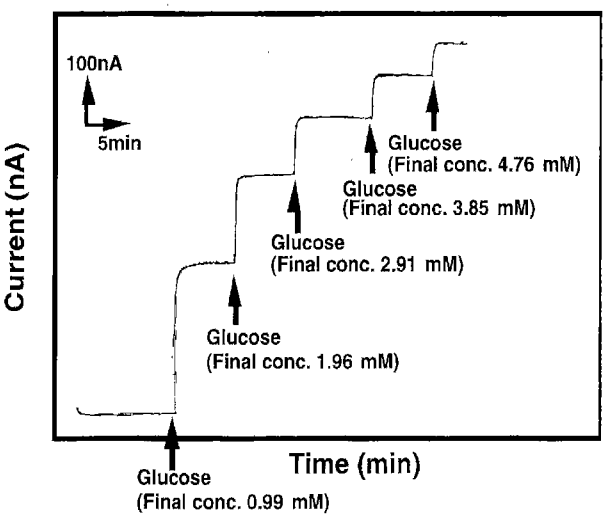

Fig. 1. Typical amperometric response to glucose addition. Buffer solution: 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM m-PMS, the operational potential: +0.25 V vs. Ag/AgCl, temperature for measurement: 25°C. Enzyme electrode was constructed with 2.5 units of the cross-linked GDH.

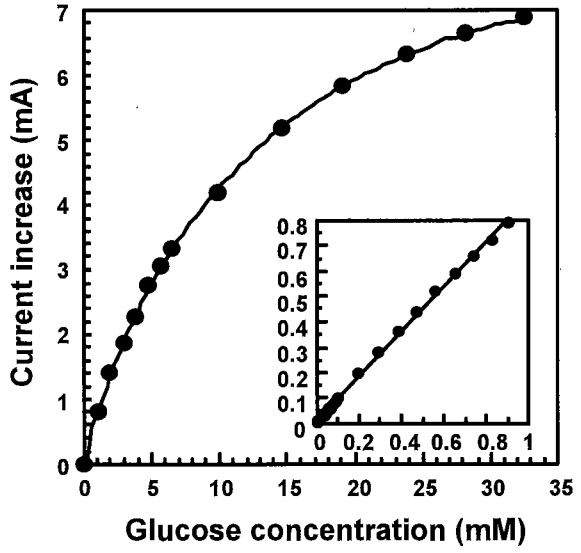

Fig. 2. Calibration curve of batch type glucose sensor. Buffer solution: 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM m-PMS, the operational potential: +0.25 V vs. Ag/AgCl, temperature for measurement: 25°C, Enzyme electrode was constructed with 2.5 units of the cross-linked GDH.

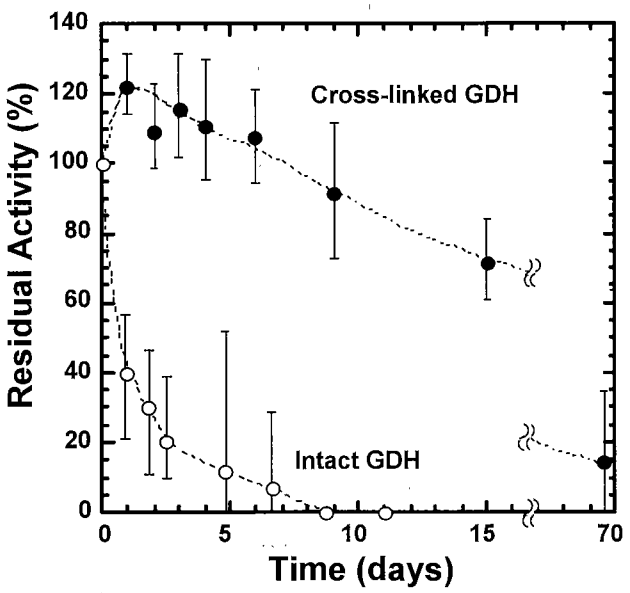

Fig. 3. Storage stability of the enzyme electrodes utilizing intact or cross-linked GDH at 50°C.
The enzyme electrodes were incubated at 50°C in the100 mM potassium phosphate buffer (pH 7.0), buffer solution for measurement: 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM m-PMS, glucose concentration: 0.99 mM, the operational potential: +0.25 V vs. Ag/AgCl, temperature for measurement: 25°C. Enzyme electrode was constructed with 2.5 units of cross-linked or intact GDH. ●cross-linked GDH, ○intact GDH.

procedure also works effectively to avoid denaturation of GDH when GDH was adsorbed in carbon paste.

3.2 The Long-term Stability of Cross-linked GDH Immobilized Enzyme Electrode This GDH acquires excellent thermal stability by cross-linking in homogeneous solution. Accordingly, the cross-linked GDH immobilized electrode may have good storage stability[15]. The Long-term stability of the enzyme electrode was tested by incubating it in 0.1 M potassium phosphate buffer (pH 7.0), at 50°C during measurement.

The cross-linked GDH immobilized electrode showed good storage stability, as shown in Figure 3. Approximately 90% of its initial response, that is 75% of its maximum response, was retained after incubation in the buffer solution for 9 days at 50°C. Even after storage at 50°C for 78 days, a calibration curve with good sensitivity was observed. On the other hand, the sensor response of the intact GDH immobilized electrode decreased quickly in the first day. After this initial period the slow decrease of the current was observed and then the intact GDH immobilized electrode lost the apparent activity within 9 days. This shows that the cross-linked GDH immobilized electrode acquired the good storage stability by cross-linking procedure to avoid thermal dissociation of GDH. Lukachova et al. previously showed thermal stabilization of enzymes immobilized into carbon paste matrix[16]. In their experiments, the enzyme electrode retained 85% of its original activity over periods of up to 4 months with thermal stress at 60°C in dry condition. While we tested the storage stability of our enzyme electrode in wet condition, it is expected to be more stable in dry condition.

3.3 Application to a FIA System An amperometric FIA system utilizing the cross-linked GDH immobilized electrode was constructed. The carrier solution was 0.1 M potassium phosphate buffer (pH 7.0), and the flow rate was 0.5 mL min^{-1}. Ferrocene was used as a mediator considering the cost for practical application. The operating potential was +0.40 V vs. Ag/AgCl. The response for duplicate injections of glucose standard solutions showed good reproducibility (Figure 4). Within 1 min after sample injections, the peak current was observed and the measurement with less than 5 min of sample injection interval was achieved. A linear relationship between the concentration of glucose and the peak current was shown up to 10 mM, and a significant increase was still observed with glucose concentration up to 50 mM (Figure 5). The lower detection limit was 0.20 mM.

3.4 Operational Stability of the Enzyme Electrode in an FIA System The operational stability was investigated in a FIA system with consecutive injections of 10 mM of glucose samples every 10 min for over 4 days. Figure 6 shows the operational stability of the sensor in the FIA system. The response decreased gradually; after 600 repetitive injections the residual response was approximately 10%, however, no significant loss in glucose sensitivity was observed during 20 repetitive injections of 10 mM of glucose. Therefore, the enzyme electrode is expected to be able to measure 600 samples when it is re-calibrated with glucose standard solution per 200 min.

In the batch analysis experiments, after storage at 50°C for 78 days, the calibration curve was obtained with good sensitivity. Considering the high storage stability at 50°C in a wet condition at batch operation analysis, the FIA was also expected to be stable for a long term continuous operation at the ambient condition. However, the sensor signals were gradually decreased by consecutive injection of the sample. It might be due to the

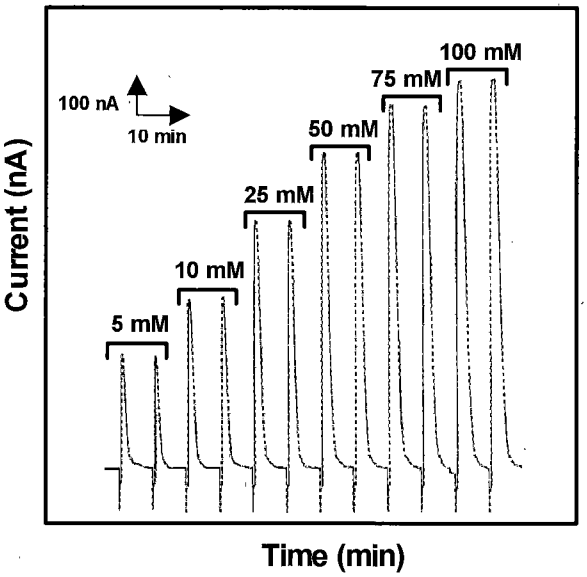

Fig. 4. Typical peaks for duplicate injections of glucose standard solutions.
Carrier: 100 mM potassium phosphate buffer (pH 7.0), flow rate: 0.5 mL min^{-1}, injection volume: 20 μL, The operational potential: +0.40 V vs. Ag/AgCl, temperature for measurement: ambient temperature, enzyme electrode was constructed with 3.0 units of the cross-linked GDH and 10 mg of ferrocene.

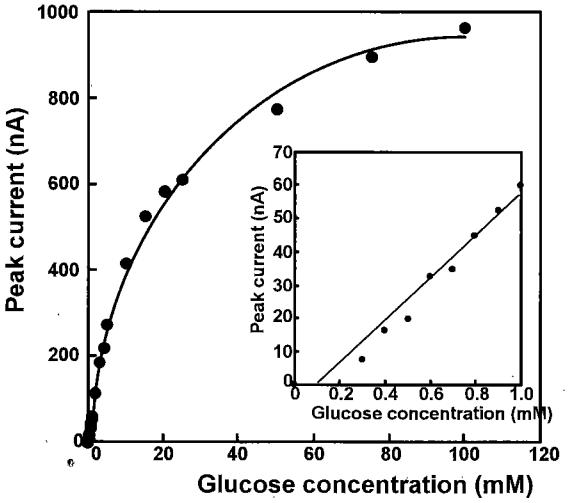

Fig. 5. Calibration curve of the FIA system.
Carrier: 100 mM potassium phosphate buffer (pH 7.0) , flow rate: 0.5 mL min^{-1}, injection volume: 20 μL, the operational potential: +0.40 V vs. Ag/AgCl, temperature for measurement: ambient temperature, nzyme electrode was constructed with 3.0 units of the cross-linked GDH and 10 mg of ferrocene.

difference in electrochemical condition between batch wise operation and FIA continuous operation. In the batch wise operation, the oxidation potential was applied only during the measurement but not during the storage. However, the FIA system was operated by continuous application of oxidation potential during whole process, including the interval of the consecutive

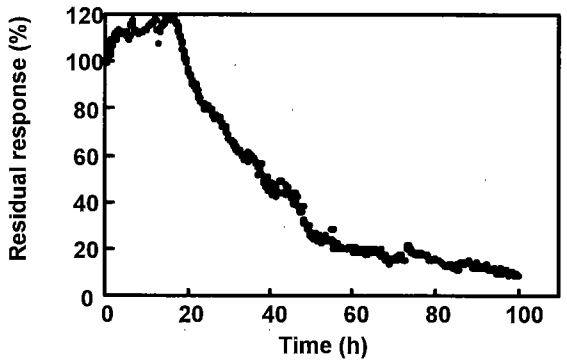

Fig. 6. Operational Stability of the FIA System.
Operational stability of the FIA system was investigated with 20 μL consecutibe injections of 10 mM glucose per 10 min. Carrier: 100 mM potassium phosphate buffer (pH 7.0), flow rate: 0.5 mL min^{-1}, the operational potential: +0.40 V vs. Ag/AgCl, temperature for measurement: ambient temperature, nzyme electrode was constructed with 3.0 units of the cross-linked GDH and 10 mg of ferrocene.

injection. The continuous application of oxidative potential might cause the denaturation of enzyme or modification of redox center of the enzyme. Parellada et al. reported the similar results in operational stability in FIA system utilizing fructose dehydrogenase and also mentioned the denaturation of the enzyme by application of potential as a reason of short operational period of the FIA system[17]. The leakage of the electron mediator from the sensor assembly should also be a factor to be considered. However, we also confirmed that the operation of FIA system by supplying mediator not in the electrode assembly but in the buffer solution (results were not shown) also caused the gradual decrease in the sensor response, as was observed in this study. Therefore, further optimization in the way of potential application of the FIA continuous system will be essential to fully appreciate the high stability of the GDH derived from the bacterial strain, SM4, as a component of FIA sensor system.

4. Conclusion

Enzyme electrode utilizing cross-linked GDH from a strain SM4 was constructed and showed good storage stability more than 70 days at 50°C. Additionally, the FIA glucose sensing system with the enzyme electrode was constructed and more than 600 consecutive measurement of the glucose was achieved.

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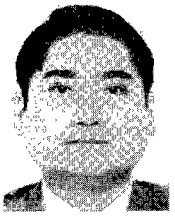

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