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Fluorescence measurement of glucose by pyrene-modified oxidase

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Abstract

Glucose oxidase was chemically modified with pyrene. The fluorescence intensity of the modified oxidase increased by glucose. The increase was attributed to the enzymatic consumption of dissolved oxygen that quenched the fluorescence of pyrene. Glucose concentration was measured quantitatively from 1 to 17 mM by the fluorescence measurement.

Keywords: Glucose; Glucose oxidase; Fluorescence; Pyrene

1. Introduction

The automated or facile determination of metabolic substrates or products is of key importance not only in clinical situations but also in industrial process, such as fermentation. Glucose is an important index in the bioreactor for the production of ethanol from raw materials containing cellulose [1] or starch [2] by using arming yeast, since glucose is a product in cellololytic pathway and a starting substance of glycolytic pathway. In order to determine the glucose concentration, the glucose electrode, which is constructed with a membrane immobilized with glucose oxidase and an oxygen electrode, have been developed and widely used [3]. Despite the usefulness of the electrodes, the other analytical methods, especially optical method, have also extensively studied [4], since the optical measurement has an advantage in the stability against electronic noise and corrosion of metallic material, and the flexibility in non-invasive measurement and telecommunication.

Upon the oxidation of glucose with glucose oxidase, oxygen is consumed and hydrogen peroxide is produced. Hydrogen peroxide can be fluorometrically detected by using peroxidase and the fluorescent substrates. Dissolved oxygen can be also fluorometrically detected, since oxygen quenches photo-excited fluorescent molecules and the fluorescence intensity decreases [5]. This mechanism has been also applied to measurements of micro environmental oxygen [6–8] and fiber optic oxygen sensor [9–12]. Here we report on a simple approach to the water-soluble fluorescent glucose sensor, based on the chemical modification of the oxidase with pyrene, which responds to glucose via the increase in fluorescence intensity based on oxygen consumption.

2. Materials and methods

Glucose oxidase was obtained from Sigma (MO, USA) and used without further purification. Glucose oxidase (76 mg) in $NaHCO_3$ solution (500 μl, pH

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9.0, 0.1 M; M = mol/dm^3) was mixed with pyrene-sulfonyl chloride (50 μl, 10 mM in acetone, obtained from Molecular Probes, OR, USA) at 4 °C for 7 h. The low molecular weight materials were removed by gel filtration (Sephadex G-10, Pharmacia, Uppsala, Sweden) and dialysis against 10 mM ammonium acetate buffer of pH 7.0. The dialysate was lyophilized and the obtained oxidase was stored in freezer. The activity of glucose oxidase was measured by using *o*-dianisidine (Wako Pure Chemical, Osaka, Japan) and horse radish peroxidase (Sigma, MO, USA). The fluorescence intensity was measured with a F700 spectrofluorometer (Hitachi, Tokyo, Japan) and monitored at 395 with 347 nm of excitation. The sample cell was heated at 35.0 °C during the measurements.

3. Results

The amount of modification was estimated 2.8 pyrene moieties per one oxidase molecule, of which value was calculated from the absorption of pyrene moiety at 357 nm and of the oxidase at 455 nm. The fluorescence spectra of the modified oxidase solution showed no excimer band, indicating that the chromophores were linked on the oxidase separately with

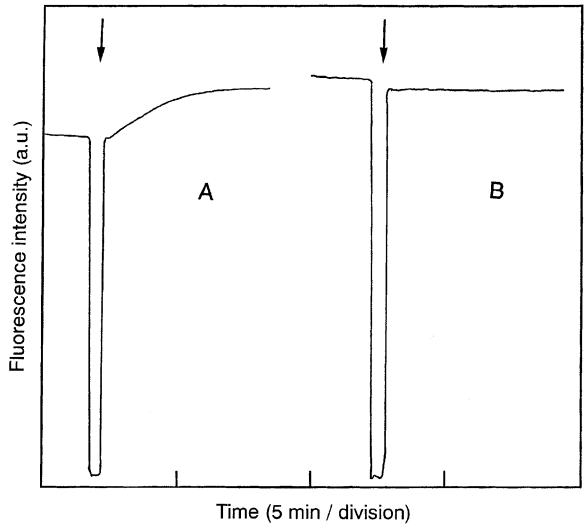

Fig. 1. Response of pyrene-modified glucose oxidase to glucose via the increase in fluorescence intensity. To the modified glucose oxidase (1.5 ml, 3.3 μg/ml, 0.1 M sodium phosphate, pH 5.5), glucose (0.56 M, 50 μl) was added at the arrow. (A) The buffer was equilibrated with air and a standard 1 cm fluorescence quartz cell was used. (B) The buffer was flushed with water-saturated nitrogen gas during the measurements and the quartz cell capped with a needle port was used. The fluorescence intensity was plotted in arbitrary unit.

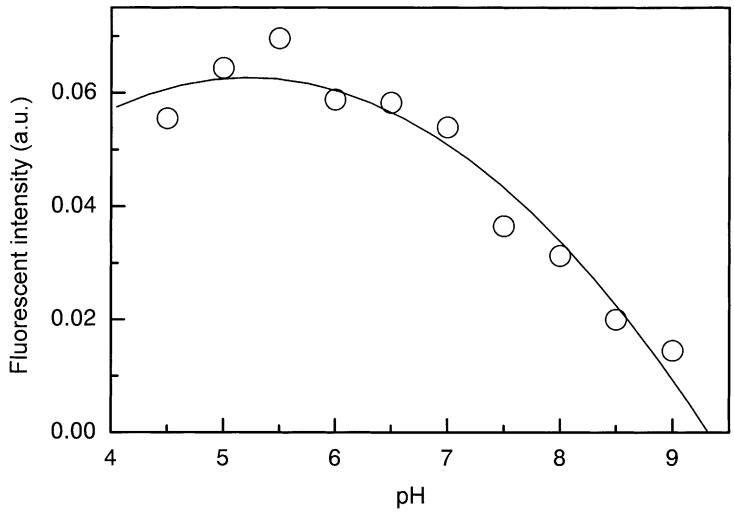

Fig. 2. Dependence of the increase of fluorescence intensity of pyrene-modified glucose oxidase on pH of the buffer solution. To the modified oxidase solution (1.5 ml, 3.3 μg/ml, 0.1 M sodium phosphate) in a standard 1 cm quartz cell, a glucose solution (0.56 M, 50 μl) was added and the time course of the fluorescence intensity was measured. The ordinate represents pH of the phosphate buffer and the abscissa represents the initial rate of the fluorescence increase in arbitrary unit.

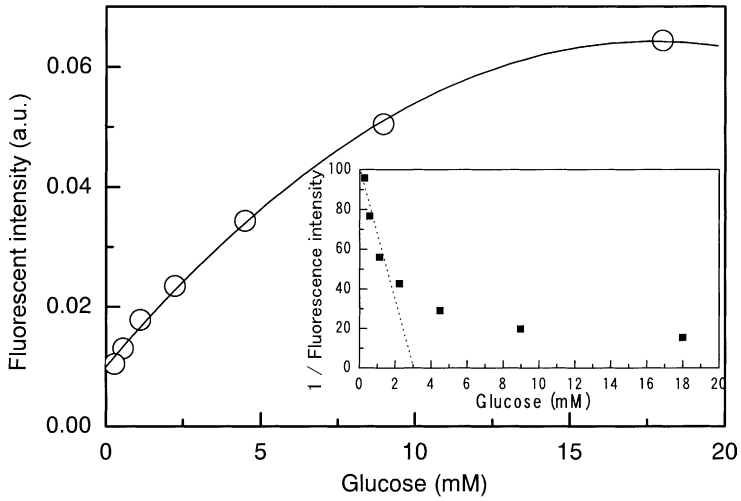

Fig. 3. Titration curve for pyrene-modified glucose oxidase based on a fluorescence change to glucose concentration. To the modified oxidase solution (1.5 ml, 3.3 μg/ml, 0.1 M sodium phosphate, pH 5.5) in a standard 1 cm quartz cell, a glucose solution (50 μl) was added and the time course of the fluorescence intensity was measured. The ordinate represents the concentration of glucose and the abscissa represents the rate of the fluorescence increase in arbitrary unit. The inset is the plot of the same data with the reciprocal of the initial rate of the fluorescence increase as the abscissa.

each other. The activity of the oxidase was ca. 60% of that of the native one.

Fig. 1A shows the fluorometric response of the modified enzyme to glucose. After the glucose was added, the fluorescence intensity increased and reached a constant intensity. Under degassed system (Fig. 1B) or the system without glucose (not shown), the increase in fluorescence intensity was not observed. When the pH of the buffer solution was varied, the fluorescence intensity changed as shown in Fig. 2.

Fig. 3 shows the dependence of glucose concentration on the fluorescence intensity. As the glucose concentration increased, the intensity increased. The dynamic range in this condition was 1.1–17.4 mM of glucose.

4. Discussion

Sulfonyl chloride compounds can react with aliphatic amines, i.e. lysine residues and N-terminal amino group to yield sulfonamide, which is stable even against acid hydrolysis, whereas possible modification of aliphatic or aromatic alcohols, thiols and imidazoles yields unstable conjugates in aqueous solution. Therefore, the most of pyrene moiety is thought to be linked to amino groups. The partial decrease of the activity by the modification is presumably due to partial denaturation during the labeling reaction by acetone, which is necessary to dissolve the sulfonyl chloride. However, the maximum value of fluorescence intensity of the pyrene-modified glucose oxidase was observed at pH 5.5 (Fig. 2), which agreed with the optimum pH value in the activity of native glucose oxidase, suggesting that the modification did not affect the catalytic site of glucose oxidase.

Glucose oxidase consumes glucose and dissolved oxygen as substrates (scheme (1)).

$$\text{glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2 \quad (1)$$

Because the fluorescence of pyrene is not so sensitive to pH change and the dependence of the buffer concentration was not observed, this increase is not attributable to pH change by gluconolactone that is produced by the oxidation of glucose. The mechanism of the fluorescence measurement would be summarized by the following schemes:

$$\text{pyr-GOD} + h\upsilon_{ex} \rightarrow \text{pyr}^*\text{-GOD} \quad (2)$$

$$\text{pyr}^*\text{-GOD} \rightarrow \text{pyr-GOD} + h\upsilon_{em} \quad (3)$$

$$\text{pyr}^*\text{-GOD} + O_2 \rightarrow \text{pyr-GOD} + O_2 \quad (4)$$

where pyr-GOD and pyr*-GOD indicate the ground state and the excited state of pyrene-modified glucose oxidase, respectively. During the fluorescence measurement, the pyrene moiety is photo-excited (scheme (2)) and emits fluorescence (scheme (3)). The photo-excited pyrene moiety competitively collides with dissolved oxygen and the fluorescence is quenched (scheme (4)) and the fluorescence $h\upsilon_{em}$ decreased. When the dissolved oxygen is consumed by the oxidase, the quenching is diminished and the fluorescence increases. The oxygen susceptivity on the fluorescence intensity is according to the Stern–Volmer relationship:

$$\frac{I_0}{I} = 1 + K\,[O_2] \tag{5}$$

where I_0 and I are the fluorescence intensity without and with quenching, respectively and K is a bimolecular quenching constant. When oxygen is consumed, Eq. (5) can be expressed as follows:

$$\frac{I_0}{I} = 1 + K\,[O_2] - K'[\text{glucose}] \tag{6}$$

where K' is a parameter for oxygen decrease arising from the Eq. (1). The plot of the reciprocal of the fluorescence intensity against the glucose concentration did not fit to a linear line (the inset of Fig. 3) and this deviation could be ascribed to the oxygen dissolution from the atmosphere.

To determine the glucose concentration for sample solutions such as bioreactor, the oxygen concentration in the solution (O_2 in Eq. (1)), which is sampled batchwise, is readily saturated with oxygen in the atmosphere. The concentration of glucose can be determined by the fluorescence intensity ($h\upsilon_{em}$ in Eq. (3)) and the calibration curve.

The oxygen consumption can be also measured by using an oxygen-sensitive fluorophore without modification but the figure of merit for the modification is easiness of measurements. Galban's group also reported the fluorescence measurement of glucose with glucose oxidase linked with 7-hydroxycoumarin-4-acetica acid [12]. The mechanism is based on the intrinsic filter effect and energy transfer based on the absorption change of FAD cofactor, and is different from the quenching by oxygen. However, the pyrene modification would be advantageously applicable to the other oxidases that consume oxygen, since the oxygen quenching is independent of the size and shape of the oxidase but the energy transfer is dependent. Marvin and Hellinga reported the fluorescent probe for glucose by using genetically engineered proteins [13]. The environmental sensitive fluorescent chromophore was incorporated at the site where the biding of glucose affects allosterically the fluorescence intensity. Our approach was facile and can be applicable to the measurements of the other substrates using oxidases that involve oxygen consumption.

In conclusion, the results reveal that concentration of glucose can be measured with the use of the chemically modified oxidase. The modified oxidase would be favored in the fabrication of optical fiber sensor because of a simple structure and also provide a prototype of a probe for two-dimensional measurement of glucose distribution.

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