Hydrogen Peroxide-Induced Deacetylation of Acetyl Resorufin as a Novel Indicator Reaction for Fluorometric Detection of Glucose Using Only Glucose Oxidase

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Hydrogen peroxide (H_2O_2)-induced deacetylation of non-fluorescent acetyl resorufin (1) to fluorescent resorufin (2) as a novel indicator reaction for fluorometric detection of glucose using only glucose oxidase (GOD) is described. When a 1 : 1 : 1 mixture of 1 (in CH_3CN), glucose, and GOD (each in pH 7.4 phosphate buffer) was incubated at 25 °C under aerobic conditions, the resulting solution turned yellow to fluorescent pink due to 2. The formation of 2 was markedly retarded on incubation under anaerobic conditions. When a mixture of 1 and H_2O_2 was incubated under aerobic conditions, the formation of 2 was noted as in the case of the enzymatic reaction of 1. These results demonstrated that the observed color change is brought about through deacetylation of 1 to 2 induced by H_2O_2 generated in GOD-catalyzed oxidation of glucose. With regard to the fluorometric traces of the enzymatic reaction with 1 (0.2 mM), GOD (0.5 mg/ml), and glucose at 25 °C, fluorescence intensity exhibited a linear relationship against glucose concentration between 0.2 and 2.0 mM, with a correlation coefficient of 0.997. Neither ascorbic acid, uric acid, nor bilirubin significantly interfered with the transformation of 1 to 2 through GOD-catalyzed oxidation of glucose.

Key words acetyl resorufin; glucose oxidase; glucose; fluorometric analysis; hydrogen peroxide; deacetylation

Spectrophotometric or fluorometric methods with glucose oxidase (GOD)–peroxidase (POD)–chromogen(s) systems are well known as useful tools for determination of glucose.[1—15] Indicator reactions in these methods are based on oxidative formation of a colored or fluorescent substance from a chromogen(s) with H_2O_2, generated through GOD-catalyzed oxidation of glucose, in the presence of POD. These color reactions are well-characterized chemical processes. However, the POD-dependent indicator reactions are known to be inevitably disturbed by electron donors present in biological samples such as ascorbic acid, uric acid, and bilirubin.[3] Major processes of interference by these compounds are as follows: 1) reduction of oxidatively formed colored or fluorescent substance to its original chromogen(s); and 2) competition with a chromogen in reduction of H_2O_2. Several methodologies have been developed for elimination of such interference in glucose determination with GOD–POD–chromogen(s) systems.[3] However, the most straightforward approach to glucose determination free from such interference is design of a novel indicator reaction without recourse to redox reactions coupled with H_2O_2 and POD. To our knowledge, only a few methods with non-redox color reactions for glucose determination using only GOD have been reported. These methods utilize transformation of a vanadium(V),[16] titanium(IV)[17] or dinuclear iron(III)[18] complex to its adduct with H_2O_2 accompanied by a bathochromic shift as a spectrophotometric indicator reaction. As expected, it was demonstrated that glucose determination with vanadium(V) and titanium(IV) complexes was not affected by various substances usually present in serum or added to test solution.[16,17] However, spectrophotometric or fluorometric determination of glucose with high accuracy should use formation of a colored or fluorescent substance from a chromogen rather than a color change of a dye as an indicator reaction.

Recently, resorufin (**2**) was shown to be able to reoxidize a reduced form of GOD at 37 °C, being transformed to a colorless dihydro derivative, although the reductive bleaching of the dye is of no use for GOD-based determination of glucose as an indicator reaction.[19] This finding prompted us to examine how resorufin derivatives such as acetyl resorufin (**1**) would behave in GOD-catalyzed oxidation of glucose, and it was found that **1** behaves in a manner different from **2** in the enzymatic reaction. Here, we report that deacetylation of non-fluorescent **1** to fluorescent **2** is induced by H_2O_2 generated in GOD-catalyzed oxidation of glucose, and the transformation is promising as an indicator reaction for fluorometric determination of glucose without significant effects of ascorbic acid, uric acid, or bilirubin.

Experimental

Materials GOD from Aspergillus niger (EC 1.1.3.4) and glucose were used as supplied from Wako Pure Chemical Industries, Ltd. Acetyl resorufin (**1**)[20—23] was prepared by reaction of resorufin sodium salt with acetic anhydride in pyridine at room temperature and recrystallized from ethyl acetate.[24] All other chemicals were of reagent grade and were used without further purification. Deionized and distilled water was used throughout the present study. CH_3CN was of HPLC grade, and a solution of **1** in CH_3CN was used throughout. All solutions of glucose, GOD, and H_2O_2 were prepared in phosphate buffer (0.1 M, pH 7.4 or 8.0; $Na_2HPO_4+NaH_2PO_4$). Glucose solutions were stored overnight to allow equilibration of α- and β-anomers.

Apparatus and Procedures During incubation of a mixture of **1** (0.1 mM in CH_3CN), GOD (0.5 mg/ml in phosphate buffer), and glucose (5.0 mM in phosphate buffer) at 25 °C under aerobic conditions, absorption spectra were obtained with a Hitachi U-3210 spectrophotometer. To obtain absorption spectra, the incubation was started by adding 1.0 ml each of solutions of **1**, GOD, and glucose to a cuvette (10×10×45 mm) in the cell holder in this order without stirring at 25 °C. All fluorometric measurements were carried out using a JASCO Model FP-750 spectrofluorometer equipped with a JASCO ETC-272 Peltier thermostatted single cell holder. Solutions of **1**, GOD, and glucose (1.0 ml each) were added to a cuvette (10×10×45 mm) in the cell holder in this order with stirring at 500 rpm and 25 °C. Measurement of the fluorescence at excitation and emission wavelengths of 568 and

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582 nm, respectively, was started 30 s after addition of glucose solution. For measurements under anaerobic conditions, each solution was added to a cuvette after Ar gas was passed through for several min, and fluorometry was performed under an Ar atmosphere. To examine the effects of ascorbic acid, uric acid, and bilirubin, fluorometric measurements were carried out under essentially the same conditions, except that a mixture of glucose and one of the additives in phosphate buffer was used instead of glucose solution. Reaction of **1** and H_2O_2 was fluorometrically traced by the same procedure after addition of 1.0 ml of a CH_3CN solution of **1** and 2.0 ml of a buffer solution of H_2O_2 to the cuvette in this order. Dissolved oxygen (DO) level was determined using a Horiba OM-14 DO meter equipped with a Horiba 5420 DO electrode. All measurements of the amount of DO in the reaction mixture were carried out by a procedure similar to that used for the fluorometric measurements except that solutions of **1**, GOD, and glucose were combined in a final volume of 30.0 ml.

Results and Discussion

A CH_3CN solution of acetyl resorufin (**1**) has a yellow color (λ_{max}; 344, 435 nm). We recently found that resorufin (**2**) with strong absorption and emission serves as an electron acceptor in GOD-catalyzed oxidation of glucose at 37 °C, being reduced to colorless dihydroresorufin.[19] Based on this finding, it was postulated that the color due to **1** would be bleached when the dye worked similarly to **2** in the enzymatic reaction. However, when a 1 : 1 : 1 mixture of **1** (0.1 mM in CH_3CN), glucose (5.0 mM in phosphate buffer), and GOD (0.5 mg/ml in phosphate buffer) was incubated at 25 °C under aerobic conditions, the mixture turned yellow to fluorescent pink. In the absorption spectrum of the mixture, a new peak with λ_{max} at 571 nm appeared as soon as the incubation was started. As incubation time was increased, the new peak became gradually higher, while the peaks due to **1** became lower (*cf.* Figs. 1a, b). The new peak at 571 nm corresponded to that of **2** in a 1 : 2 solution of CH_3CN–phosphate buffer. The absorbance of the new peak observed after incubation for 45 min (Fig. 1b) was markedly larger than that observed after incubation of **1** in the absence of glucose for 45 min (Fig. 1c). These results demonstrated that the color change observed in the incubation of a mixture of **1**, glucose, and GOD is due to transformation of **1** to **2**, which is for the most part induced through the enzymatic reaction (Chart 1). It should be noted here that **2** does not function as an electron acceptor in GOD-catalyzed oxidation of glucose at 25 °C. Therefore, a fluorescent pink coloration due to the transformation of **1** into **2** through the enzymatic reaction was not bleached by reduction of newly formed **2** to its dihydro derivative by a reduced form of GOD. Enzymatic consumption and release of **2** have been demonstrated to function as useful indicator reactions for spectrophotometric and fluorometric analyses, since the dye exhibits strong absorption (λ_{max} 571 nm, ε 4—7$\times 10^4$) and emission (excitation maximum at 563 nm and emission maximum at 587 nm at pH 7.4) at wavelengths$>$550 nm, where potential interference in analysis of colored or turbid serum components can be avoided.[25—35] These observations imply that the transformation of **1** to **2** as an indicator reaction for glucose analysis using only GOD takes full advantage of **2** as an analytically useful dye.

The reaction of **1**, glucose, and GOD was studied in more detail by a fluorometric method. Enzymatic reaction of **1**, glucose, and GOD was initiated by addition of glucose to a mixture of **1** and GOD, where each of the solutions was combined in the same volume, usually 1.0 ml. All fluorometric measurements were carried out with 568 and 582 nm as excitation and emission wavelengths, respectively, and were set from 30 s after initiation of the enzymatic reaction. To establish useful conditions for transformation of non-fluorescent **1** to fluorescent **2** as an indicator reaction for analysis of glucose, effects of the concentrations of **1** and GOD on fluorometric changes in the present enzymatic reaction were determined. Figure 2 summarizes the effects of the concentration of **1** (in CH_3CN) on fluorometric changes during the enzymatic reaction with 1.0 mM glucose (in phosphate buffer) and 2.0 mg/ml GOD (in phosphate buffer). A progression curve with a larger change in fluorescence intensity was observed as the concentration of **1** was increased between 0.05 and 0.3

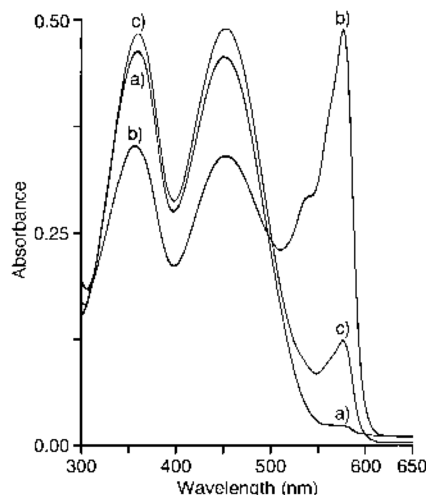

Fig. 1. Absorption Spectra Obtained Immediately (a) and after 45 min (b) incubation of a 1 : 1 : 1 Mixture of **1** (0.1 mM in CH_3CN), Glucose (5.0 mM in pH 7.4 Phosphate Buffer), and GOD (0.5 mg/ml in pH 7.4 Phosphate Buffer) at 25 °C under Aerobic Conditions, and That (c) Obtained after 45 min Incubation of a 1 : 1 : 1 mixture of **1** (0.1 mM in CH_3CN), a Blank Solution (pH 7.4 Phosphate Buffer), and GOD (0.5 mg/ml in pH 7.4 Phosphate Buffer) under the Same Conditions

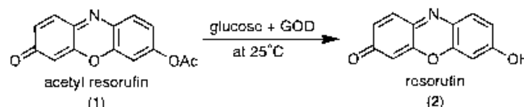

Chart 1

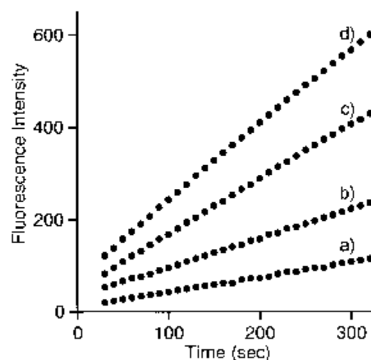

Fig. 2. Effects of Concentration of **1** on Fluorometric Traces Obtained during Incubation of a 1 : 1 : 1 Mixture of **1** (in CH_3CN), Glucose (1.0 mM in pH 7.4 phosphate buffer), and GOD (2.0 mg/ml in pH 7.4 Phosphate Buffer) at 25 °C for 5.5 min under Aerobic Conditions

Concentration of **1** in the stock solution was varied between 0.05 (a), 0.1 (b), 0.2 (c), and 0.3 mM (d). Excitation and emission wavelengths were 568 and 582 nm, respectively.

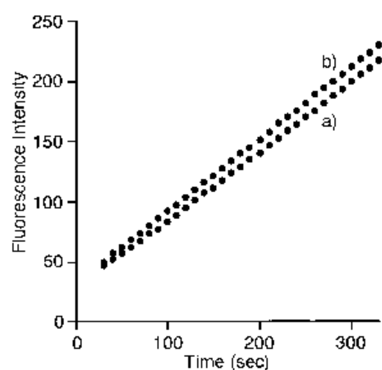

Fig. 3. Effects of Concentration of GOD on Fluorometric Traces Obtained during Incubation of a 1 : 1 : 1 Mixture of **1** (0.1 mM in CH_3CN), Glucose (1.0 mM in pH 7.4 Phosphate Buffer), and GOD (in pH 7.4 Phosphate Buffer) at 25 °C for 5.5 min under Aerobic Conditions

Concentration of GOD in the stock solution was varied between 0.5 (a) and 4.0 (b) mg/ml. Excitation and emission wavelengths were 568 and 582 nm, respectively.

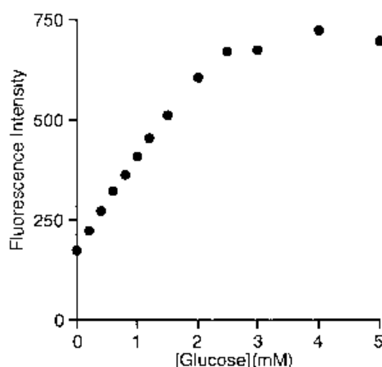

Fig. 4. Relationship of Glucose Concentration with Fluorometric Intensity Observed after 5 min Incubation of a 1 : 1 : 1 Mixture of **1** (0.2 mM), Glucose (in pH 7.4 Phosphate Buffer), and GOD (0.5 mg/ml in pH 7.4 Phosphate Buffer) at 25 °C under Aerobic Conditions

The spectrophotometric responses were obtained using 568 and 582 nm as excitation and emission wavelengths, respectively.

mM. As can be seen in Fig. 3, which includes fluorometric traces obtained with only 0.5 and 4.0 mg/ml GOD for simplicity, progression curves observed in the reactions with 0.1 mM **1** and 1.0 mM glucose could nearly be superimposed regardless of GOD concentration. Thus, it is likely that the rate of transformation of **1** to **2** through the enzymatic reaction is not influenced by GOD concentration, at least in the range between 0.5—4.0 mg/ml.

The possibility of transformation of **1** to **2** as a fluorometric indicator reaction in glucose detection was then explored. Based on the detection limit of the fluorometer as well as the above results, a CH_3CN solution of **1** in 0.2 mM and a buffer solution of GOD in 0.5 mg/ml were utilized for this purpose. In Fig. 4, fluorescence intensity observed 5 min after initiation of the enzymatic reaction on a mixture of **1**, glucose, and GOD is plotted against the concentration of glucose. A linear relation was found between the intensity and the glucose concentration of 0.2—2.0 mM, with a slope and a correlation coefficient of 216.9/mM and 0.997, respectively. Relative standard deviations (RSD) (n=3) for fluorescent responses obtained with glucose in 0.2 and 2.0 mM were 2.5 and 0.5%, respectively. In addition, the fluorescence intensity observed after 5 min incubation in the absence of glucose was also reproducible, RSD (n=3) being 4.8%. When glucose at a concentration of more than 2.5 mM was subjected to the enzymatic reaction, fluorescence intensity obtained after 5 min reached an almost constant value, independent of glucose concentration.

The transformation of **1** to **2** was also found to serve as a fluorometric indicator reaction in glucose detection when the enzymatic reaction was carried out using solutions of glucose and GOD (0.5 mg/ml) in pH 8.0 phosphate buffer. Under these conditions, fluorometric responses became larger and a CH_3CN solution of **1** in 50 μM instead of 0.2 mM was used. A relationship similar to that in Fig. 4 was recognized between the glucose concentration and fluorescence intensity observed 5 min after initiation of the enzymatic reaction. However, a linear relation was observed only over the range of glucose concentration between 0.2 and 1.5 mM. Thus, the working range for glucose detection with the present indicator reaction was slightly wider at pH 7.4 than at pH 8.0.

The effects of ascorbic acid, uric acid, and bilirubin on this reaction for glucose determination were evaluated. For this purpose, fluorescence intensity obtained 5 min after initiation of incubation of a mixture of **1** (0.2 mM in CH_3CN), GOD (0.5 mg/ml in pH 7.4 phosphate buffer), glucose (1.0 mM in pH 7.4 phosphate buffer), and one of the additives was compared with that obtained without the additive. The incubation was initiated by addition of a phosphate buffer solution containing both glucose and the additive to a mixture of **1** and GOD in a fluorometric cell under aerobic conditions. The results are summarized in Table 1. The examined compounds at concentrations equivalent to or even higher than those in normal serum showed no significant influence on the transformation of **1** to **2** induced by GOD-catalyzed oxidation of glucose. These results are reasonable since the present GOD–chromogen system provides an indicator reaction based on non-oxidative formation of a fluorescent substance.

To determine the mechanism responsible for the enzymatically induced transformation of **1** to **2**, a mixture of **1** (0.2 mM in CH_3CN), GOD (0.5 mg/ml in pH 7.4 phosphate buffer), and glucose (1.0 mM in pH 7.4 phosphate buffer) was incubated at 25 °C under anaerobic conditions, which was followed by essentially the same fluorometric method. The trace obtained under anaerobic conditions is compared with that afforded under aerobic conditions in Fig. 5. The fluorometric trace observed for incubation of **1** with only GOD under aerobic conditions is also included in the figure. During the enzymatic reaction under anaerobic conditions, the change in fluorescence intensity due to transformation of **1** to **2** was markedly smaller than that under aerobic conditions. Although incubation under anaerobic conditions gave a progression curve with a slightly greater slope than incubation of the blank solution under aerobic conditions, it was clear that DO plays an important role in deacetylation of **1**.

In the present enzymatic reaction, DO was rapidly consumed in a manner dependent on glucose concentration, when a mixture of **1**, GOD, and glucose was subjected to incubation at 25 °C under aerobic conditions. This observation implied that H_2O_2 formed from DO through the enzymatic reaction induces the transformation of **1** to **2**. When reaction of **1** (0.2 mM in CH_3CN) with H_2O_2 itself (0, 0.22, 0.44, 0.66, and 0.88 mM in pH 7.4 phosphate buffer) was followed by a

Table 1. Effects of Some Organic Substances on the Fluorescence Intensity Observed after 5 min Incubation of a Mixture of **1** (0.2 mM in CH_3CN), GOD (0.5 mg/ml in pH 7.4 Phosphate Buffer), and Glucose (1.0 mM in pH 7.4 Phosphate Buffer) (1.0 ml Each)[a]

Substance	Concentration added (mM)[b]	Relative intensity (%)[c]
Ascorbic acid	0.2	99.9
	0.4	97.8
Uric acid	0.2	99.4
	0.4	99.5
Bilirubin	0.02	101.8
	0.04	100.2

a) Excitation and emission wavelengths were 568 and 582 nm, respectively. *b*) Concentration in the stock solution. *c*) Ratio of the fluorescence intensities obtained in the presence of additive against those obtained without the additive.

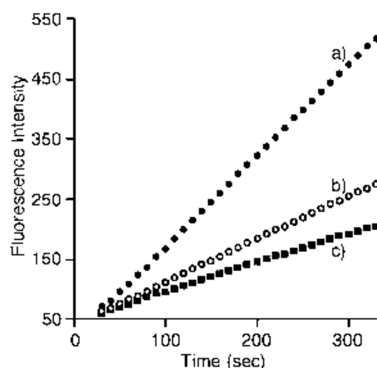

Fig. 5. Fluorometric Traces Obtained during Incubation of a 1 : 1 : 1 Mixture of **1** (0.2 mM in CH_3CN), Glucose (1.0 mM in pH 7.4 Phosphate Buffer), and GOD (0.5 mg/ml in pH 7.4 Phosphate Buffer) at 25 °C for 5.5 min under Aerobic (a) and Anaerobic (b) Conditions, and That (c) Obtained during Incubation of a 1 : 1 : 1 Mixture of **1** (0.1 mM in CH_3CN), a Blank Solution (pH 7.4 Phosphate Buffer), and GOD (0.5 mg/ml in pH 7.4 Phosphate Buffer) under Aerobic Conditions

Excitation and emission wavelengths were 568 and 582 nm, respectively.

similar fluorometric method, progression curves were obtained, with increments depending on H_2O_2 concentration, as depicted in Fig. 6. It has been proposed that nucleophilic addition of H_2O_2 to aryl oxalate esters results in formation of the corresponding phenols and 1,2-dioxetane.[36—38] According to this mechanism, nucleophilic substitution by H_2O_2 is thought to be responsible for transformation of **1** to **2** during GOD-catalyzed oxidation of glucose, which is much faster than simple hydrolysis of **1** in CH_3CN–buffer solution. It should be noted here that when glucose at a concentration of more than 2.5 mM was incubated under aerobic conditions, DO was totally consumed, suggesting that the amount of H_2O_2 generated was constant. This is why a plateau region was observed in the relationship between fluorescence intensity and glucose concentration as shown in Fig. 4.

In conclusion, it was demonstrated that in GOD-catalyzed oxidation of glucose, deacetylation of **1** occurs yielding **2** in a manner dependent on glucose concentration without significant effects of ascorbic acid, uric acid, or bilirubin, and hence the reaction can be utilized as a novel indicator reaction for fluorometric detection of glucose using only GOD. As mentioned above, several analytical methods with spectrophotometry or fluorometry have been designed based on enzymatic release of **2** from its derivatives such as **1**.[25—35] However, to our knowledge, generation of **2** from **1** has been used as an indicator reaction only for studies of esterase activity of cytosolic aldehyde dehydrogenase and chymotrypsin.[20—23] Thus, this is the first report to show that transformation of **1** to **2** is induced by H_2O_2. Although practical application of the present indicator reaction requires prevention of simple hydrolysis of **1** in blank solution, the present results suggested some intriguing points from the standpoint of enzymatic analysis of biological compounds, as follows: 1) H_2O_2-induced deacylation of **1** and other acylated **2** will find applications as indicator reactions for enzymatic analysis of various biological compounds based on oxidoreductases with oxygen as an electron acceptor such as GOD, cholesterol oxidase, uricase, *etc.*; 2) leuco dyes, produced by acylation of phenolic hydroxy groups of dyes, might be deacylated by H_2O_2, which process can be used as a novel indicator reaction for POD-independent enzymatic analysis, similarly to deacylation of **1** and other acylated **2**. Further studies are currently underway in our laboratory to examine these points.

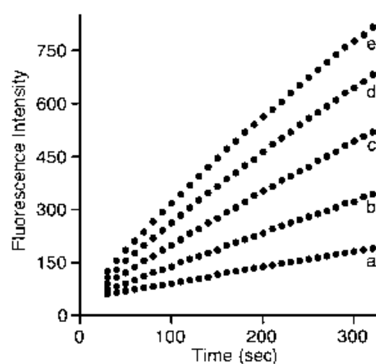

Fig. 6. Fluorometric Traces Obtained during Incubation of a 1 : 2 Mixture of **1** (0.2 mM) and H_2O_2 (in pH 7.4 phosphate buffer) at 25 °C for 5.5 min under Aerobic Conditions

Concentration of H_2O_2 in the stock solution was varied between 0 (a), 0.22 (b), 0.44 (c), 0.66 (d), and 0.88 mM (f). Excitation and emission wavelengths were 568 and 582 nm, respectively.

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