Immobilized Enzyme-Based Microtiter Plate Assay for Glucose in Foods

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The method presented is based on the use of glucose oxidase (GOD), peroxidase (POD), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Enzymes were separately immobilized on the inside surface of each well of a 96-well microtiter plate. Prior to the immobilization, the surface was activated with glutaraldehyde. The activation conditions remarkably affected both the activity and the stability of immobilized enzymes. A solution containing H_2O_2 (20 μL) was added into the well with immobilized POD (POD-well) containing 180 μL of 2 mM ABTS (pH 6.5) and the absorbance change at 415 nm for 10 min was measured with a microplate reader. A sample solution containing D-glucose was first added into the well with immobilized GOD. After 30 min, an aliquot (20 μL) was transferred into the POD-well. The calibration curves for H_2O_2 and D-glucose were linear up to 1 and 1.5 mM, respectively. The glucose concentration in fruit juices obtained by the present method agreed well with that by the F-kit method.

Keywords: *Glucose; glucose oxidase; peroxidase; immobilized enzyme; microtiter plate assay; citrus; fruits juice*

INTRODUCTION

Immobilized enzymes are increasingly used as tools in analytical chemistry, especially as enzyme electrodes in flow-systems such as flow injection analysis (FIA) or in combination with HPLC as a post-column reaction system (Schwedt and Stein, 1994). The electrode-type and FIA system based on immobilized enzyme have been extensively developed for application in food analysis (Bilitewski, 1994; Lopez-Fernandez et al., 1995; Ukeda, 1995). A combination of FIA with immobilized enzymes is especially attractive because of two important features: (a) the high substrate selectivity and sensitivity of enzymes and (b) the rapid and automated handling of liquids by FIA.

Sampling frequency of up to 60 samples/h has been achieved (Bilitewski, 1994). However, the frequency may still be unsatisfactory in some food industries because quality control sometimes needs much higher sampling frequency. Also, the FIA systems developed so far seem to be impractical for the simultaneous determination of multicomponents in food samples because the instrumental compositions become relatively more complicated as the number of components under analysis increases (Matsumoto et al., 1990; Yoshioka et al., 1992; Lee et al., 1996).

We propose here a novel method of food analysis based on immobilized enzymes in which a 96-well microtiter plate with immobilized enzymes is used as the integrated reaction vessel. The 96-well microtiter plate has been widely used for enzyme-immunometric assays (EIA) and the configuration is suitable for integrating the reaction vessel with immobilized enzymes. Also, a spectrophotometric microplate reader capable of quickly and simultaneously detecting a colorimetric change in the plate has also come into wide use in many laboratories. Therefore, this combination would be potentially attractive for the simultaneous determination of multisamples and multicomponents of food.

For the purpose of establishing the principle of an immobilized enzyme-based microtiter plate assay, in the present investigation, a method of determining D-glucose was developed and applied to the analysis of D-glucose in practical food samples. The method is based on the enzyme reaction of glucose oxidase (GOD) and peroxidase (POD), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is used as electron donor for POD (Kunst et al., 1984; Putter and Becker, 1984):

$$\beta\text{-D-glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} 2H_2O + \text{ABTS (oxidized form)}$$

GOD and POD were separately immobilized on the inside surface of each well. An attempt was made to improve the stability of the immobilized enzyme by controlling the reaction conditions of glutaraldehyde used for activation of the well.

MATERIALS AND METHODS

Materials. POD (EC 1.11.1.7; 1000 units/mg; from horseradish; for EIA grade) and ABTS were purchased from Boehringer Mannheim (Mannheim, Germany). GOD (EC 1.1.3.4; 100 units/mg; from *Aspergillus* sp.) was obtained from Amano Pharmaceutical Company (Nagoya, Japan). Glutaraldehyde (70% aqueous solution) was purchased from Taab Laboratories Equipment (Berks, U.K.). The microtiter plate with primary amino group (plate A) and the polystyrene microtiter plate without a special functional group (plate B) were purchased from Coaster (Cambridge, MA), and the plate with secondary amino group (CovaLink; plate C) was obtained from Nunc (Roskilde, Denmark).

Modification of POD. The sugar chain of POD was modified according to Nakane's method (Wilson and Nakane, 1978) with a slight modification: POD (2 mg dissolved in 1

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mL of water) was oxidized with 0.1 mL of 100 mM $NaIO_4$ for 20 min at room temperature and dialyzed against 1 mM acetate buffer (pH 4.4) at 4 °C ($NaIO_4$-oxidized POD). To the solution was added 1 mL of 0.2 M carbonate buffer containing 15 mg of 1,6-diaminohexane. After 2 h, 0.1 mL of $NaBH_4$ solution (4 mg/mL) was added to reduce the Schiff base. The reaction mixture was kept at 5 °C for 2 h and again dialyzed against 0.1 M potassium phosphate buffer (pH 6.5). This solution was used for the immobilization to the microtiter plate as the aminated POD. The concentration of POD was calculated from the absorbance at 403 nm.

Immobilization of POD. The following three methods were examined. (a) Each well of the microtiter plate was activated with glutaraldehyde solution (200 μL) prepared with 0.2 M carbonate buffer (pH 10) for a given time at 22 ± 1 °C. After thoroughly washing the well with 0.1 M phosphate buffer (pH 6.5), 200 μL of the buffer (pH 6.5) containing 25 μg of the aminated POD was added into the well. After the immobilization reaction for 18 h at 5 °C, the unreacted POD was removed by washing thoroughly with the buffer. (b) Unmodified POD was directly immobilized to glutaraldehyde-activated well by using unmodified POD instead of aminated POD in method (a). (c) $NaIO_4$-oxidized POD was added into each well of plate A. After reaction for 18 h, it was treated with $NaBH_4$ (4 mg/mL) for 2 h at 5 °C. The three plates were stored at 5 °C in 0.1 M phosphate buffer (pH 6.5) when not in use.

Immobilization of GOD. The principle of this method was the same as that of method (a) of POD just described except that native GOD was used. The well was activated by reaction with 10% glutaraldehyde in 0.2 M carbonate buffer (pH 10) for 20 h at 37 °C, unless otherwise mentioned. The GOD solution for coupling was prepared with 0.1 M phosphate buffer (pH 6.5) and the solution contained 360 μg of GOD in 200 μL. The plate was stored at 5 °C, and the coupling buffer was filled into it when not in use.

Determination of H_2O_2. The H_2O_2 solution (20 μL) was added into each well with immobilized POD (POD-well) containing 180 μL of 2 mM ABTS prepared with 0.1 M phosphate buffer (pH 6.5). After mixing at 500 rpm with a microplate mixer at 22 ± 1 °C, the absorbance at 415 nm based on the formation of oxidized form of ABTS was measured with a microplate reader (MPR A4i, Tosoh, Tokyo, Japan). The reaction mixture in the well was aspirated and washed with the same buffer at least three times immediately after the measurement. The concentration of the standard H_2O_2 solution was calibrated by the absorbance at 240 nm (43.6 M^{-1} cm^{-1}) or by iodometry.

Determination of D-Glucose. The standard and sample solutions (200 μL) were added into the well with immobilized GOD (GOD-well). After a given time, an aliquot (20 μL) of the reaction solution was transferred into the POD-well that already contained 180 μL of 2 mM ABTS. The absorbance at 415 nm after 10 min was measured. The standard solution of D-glucose was prepared with 0.1 M phosphate buffer (pH 6.5). The well was washed with the sampling buffer. Prior to analysis of D-glucose, commercially available fruit juices and freshly squeezed orange juice were diluted 200- to 400-fold with 0.1 M phosphate buffer (pH 6.5). The concentration of D-glucose in these practical samples was calculated from the calibration curve of the standard D-glucose solution.

Determination of D-Glucose by the F-Kit Method. The F-kit for D-glucose was purchased from Boehringer Mannheim, and the analyses were performed according to the manufacturer's manual. The F-kit determines D-glucose by an enzymatic, spectrophotometric method based on the use of hexokinase, glucose-6-phosphate dehydrogenase, and NADP.

RESULTS AND DISCUSSION

Immobilization Method of POD. The three immobilization methods of POD were compared with plate A. The absorbances in each well due to the reaction of 1 mM H_2O_2 for 40 min are shown in Figure 1. When an excessive amount of free POD was added into the well, the reaction mixture showed an absorbance of 2.5 within 5 min. The POD-well prepared by method b had only a small increase in the absorbance during the reaction time and a rapid decrease in the activity during storage. Method b was based on a general principle for enzyme immobilization using glutaraldehyde (Ukeda et al., 1995). The coupling of enzyme to a glutaraldehyde-activated support is considered to be due to the reaction of the amino group on an enzyme molecule with the aldehyde group to form a Schiff base. According to Welinder and Mazza (1977), six ϵ-amino groups of a POD molecule are covered with sugar chains. Therefore, this result may suggest that those amino groups of POD were not available for coupling with the glutaraldehyde-activated well.

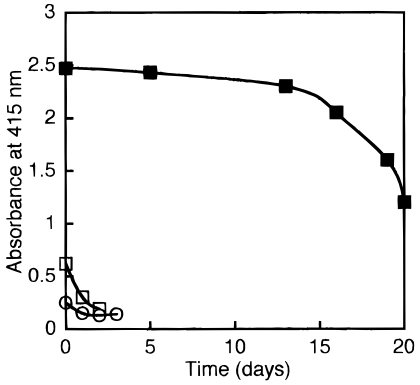

Figure 1. Activity and storage stability of POD immobilized by different methods. POD was immobilized by method a (■), method b (□), and method c (○). Each activity of the immobilized POD is shown as the absorbance by the reaction with 1 mM H_2O_2 for 40 min.

Next, we examined the applicability of the sugar chain to the coupling. In method c, the sugar chain was oxidized with $NaIO_4$ to form an aldehyde group, and the functional group was used for coupling with an amino group on the well, followed by the reduction with $NaBH_4$. As shown in Figure 1, however, the well indicated a smaller increase in the absorbance. As the $NaIO_4$-oxidized POD retained the activity, this was perhaps because of too small an amount of the immobilized POD. Another possibility is inactivation of POD by the immobilization procedure. POD is known to be inactivated by the interaction with a plastic interface, such as a microtiter plate (Berkowitz and Webert, 1981). In EIA, a detergent such as Tween 20 is usually added into the assay solution to prevent the inactivation. In this method, there was no spacer molecule between the immobilized POD and the inside surface of the plate. Therefore, the immobilized POD might have been inactivated by the hydrophobic interaction even if enough POD was immobilized by the method. It was assumed from these results that modifications of having enough functional groups into the POD molecule and the introducing a spacer between the enzyme and the plate would be needed to prepare active immobilized POD. In method a, the plate was activated with glutaraldehyde in the role of the spacer, and amino groups were introduced by the reaction of $NaIO_4$-oxidized POD with excessive amount of 1,6-diaminohexane and by the subsequent reduction. The POD-well prepared by method a showed much higher activity than methods b and c (Figure 1). The well immediately after the immobilization reaction showed an absorbance change of 2.5, meaning that all of the added H_2O_2 was reduced within 40 min. In addition, the decrease in the activity of the well during the storage was relatively small. It was judged that the

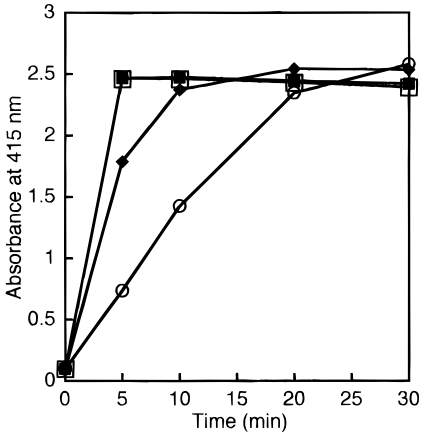

Figure 2. Time course of the reaction with 1 mM H_2O_2 of POD-well prepared under different activation conditions with glutaraldehyde. The concentrations and the reaction times of glutaraldehyde used in activation of each well were as follows: (○) 5% for 4 h; (◆) 5% for 8 h; (■) 10% for 4 h; (□) 10% for 8 h.

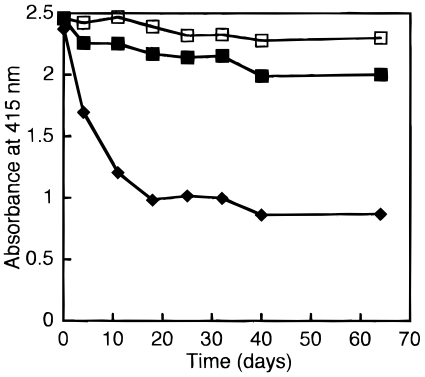

Figure 3. Stability of POD-well prepared under different activation conditions with glutaraldehyde. The concentrations and the reaction times of glutaraldehyde used in activation of each well were as follows: (◆) 5% for 8 h; (■) 10% for 4 h; (□) 10% for 8 h. Each point shows the absorbance by the reaction with 1 mM H_2O_2 for 40 min.

principle of the method was suitable for the immobilization of POD to the microtiter plate and the conditions were, therefore, examined further.

The effect of the activation conditions of the microplate with glutaraldehyde on the activity of the immobilized POD are depicted in Figure 2. The time to reach at the absorbance corresponding to the endpoint was shortened with an increase in the concentration and reaction time of glutaraldehyde. The well activated with 10% glutaraldehyde solution (pH 10) for 4 or 8 h showed the highest activity, and the absorbance was reached at the endpoint within 5 min. The stability of these wells are shown in Figure 3. The stability also depended on the reaction conditions of glutaraldehyde to a great extent, and the immobilized POD prepared with higher concentration and longer reaction time of glutaraldehyde showed a higher stability. The well activated with 10% glutaraldehyde solution (pH 10) for 8 h was the most stable and showed no significant decrease in the activity for 2 months. From these results, the activation conditions of 10% glutaraldehyde for 8 h were used as the optimum for immobilization of POD in a subsequent experiment.

In this experiment, a buffer of pH 10 was used for the activation with glutaraldehyde. Glutaraldehyde is believed to undergo aldol condensations to form α,β-unsaturated aldehydes at such an alkaline pH (Margel and Rembaum, 1980). Dobbins Place and Schroeder (1982) found that the polymerized glutaraldehyde formed by aldol condensation played a major role in the binding of hepatitis B surface antigen to the plastic surface of the microtiter plate and the monomer was ineffective. Generally, the polymerization reaction occurs more quickly with an increase in concentration, and the degree of polymerization increases with increasing reaction time (Rasmussen and Albrechtsen, 1974). Therefore, the polymerized form could be considered to be more effective for the immobilization of POD, as well as in the binding of hepatitis B surface antigen.

Three kinds of the microplates (plates A, B, and C) were activated with glutaraldehyde under the conditions just described to immobilize POD. Those activities just after the preparation were all high enough for the absorbance to reach the endpoint within 10 min. There was also no significant difference in the stability. This result indicates that the activation reaction with glutaraldehyde is independent of the structure of the surface of the plate, and the functional group (such as amino group) that can bind with aldehyde group is not necessarily required because plate B without any specific functional group for binding had an almost equal activity and stability. Thus, the polymerized form of glutaraldehyde effective for the immobilization of POD may bind to the surface by hydrophobic interaction. Plate C had a slightly higher stability, so this microplate was used for a subsequent experiment.

Optimization of H_2O_2 Determination. The dependence of the concentration of ABTS was examined with 1 mM H_2O_2. The absorbance increased with an increase in the concentration up to 0.5 mM, and a tendency to level off was recognized above this concentration.

The effect of pH on the absorbance for 1 mM H_2O_2 was studied in the pH range of 5.5 to 8.0. There was no significant difference in the initial rate of the absorbance change between pH values, but the pH optimum in the free state was 6.0−6.5, as previously reported (Putter and Becker, 1984). The absorbance at each endpoint decreased slightly with increasing reaction pH. This decrease could be caused by instability of the oxidized form of ABTS (ABTS radical cation) at alkaline pH (McCoy-Messer and Bateman, 1993).

The effect of the addition of Tween 20 (0.05%) on the determination of H_2O_2 was investigated with the reaction buffer in the pH range 5.5−8.0. As described earlier, Tween 20 is often added to the assay solution of POD to prevent the inactivation by contact with hydrophobic surface of microplate in EIA. Compared with the initial rate where no addition of Tween 20 was made, the rate increased ~1.5-fold at all pH levels. However, the absorbance at the endpoint remarkably decreased with increasing pH, indicating that the ABTS radical cation was less stable in the presence of Tween 20.

From these results, 0.1 M potassium phosphate buffer (pH 6.5) and 2 mM ABTS were chosen as optimum conditions. The absorbance reached the endpoint within 5 min, so the value after 10 min was measured. Under these conditions, the calibration curve for H_2O_2 was linear in the range 0.05−1 mM, with a correlation coefficient of 0.999 [$y = 2.27x + 0.09$; y, absorbance at 415 nm; x, H_2O_2 concentration (mM)]. The relative standard deviation for 1 mM H_2O_2 by eight successive determinations with an identical well was <1%. Also, the relative standard deviations of eight different POD-

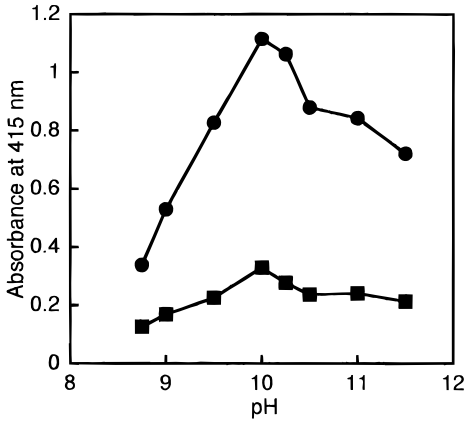

Figure 4. Effect of activation pH with glutaraldehyde on the activity of immobilized GOD. Each point shows the absorbance by the reaction with 1 mM (●) and 0.2 mM (■) D-glucose for 30 min.

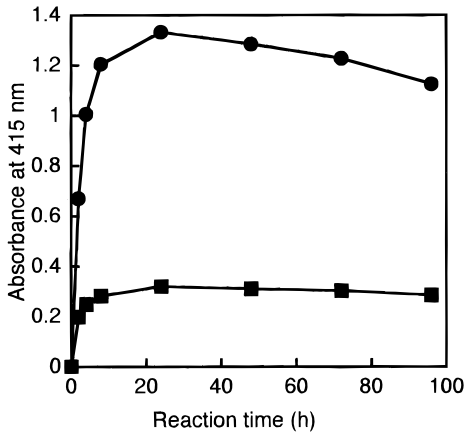

Figure 5. Effect of activation reaction of glutaraldehyde on the activity of immobilized GOD. Each point shows the absorbance by the reaction with 1 mM (●) and 0.2 mM (■) D-glucose for 30 min.

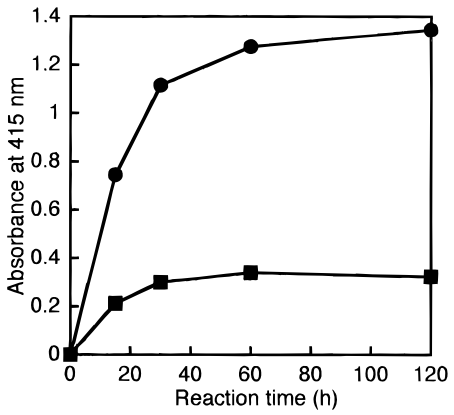

Figure 6. Time course of the reaction with 1 mM (●) and 0.2 mM (■) D-glucose by GOD-well prepared under optimum conditions.

wells were 1.8 and 4.1% for 1.0 and 0.2 mM H_2O_2, respectively. The storage stability of the POD-well was relatively good, and a constant absorbance for 1 mM H_2O_2 was recognized during the storage for 350 days. Thirty repetitive determinations of 1 mM H_2O_2 did not affect the activity of the plate. However, a gradual decrease in the absorbance change for 10 min was recognized in the subsequent determinations.

Optimization of Determination of D-Glucose in Standard Solution. The optimum conditions for the immobilization of POD were applied to the immobilization of GOD. However, the oxidation reaction of D-glucose with the immobilized GOD was very slow, and the absorbance did not reach at the plateau even after 3 h. From this result, we re-examined the optimum conditions for the GOD immobilization, including the activation reaction with glutaraldehyde.

As the formation of polymerized glutaraldehyde during the activation reaction was important to obtain higher activity of the immobilized POD, the reaction conditions of glutaraldehyde that affected its formation were studied; for examples, the concentration, the reaction time, and temperature. When the temperature was set at 37 °C, the oxidation reaction of the GOD-well was accelerated 1.5-fold compared with the GOD-well activated at 22 °C. As the reaction rate of polymerization increases by a factor of three with a temperature increase of 10 °C (Rasmussen and Albrechtsen, 1974), the activity of the immobilized GOD could be clearly considered to have increased with the increase of the degree of polymerization. The effect of the reaction pH of glutaraldehyde on the activity of the immobilized GOD is shown in Figure 4, where the values indicate the absorbance change after 30 min. The activity increased with raising the pH of activation in the range 8.7−10.0, followed by a gradual decrease above pH 10. The dependence of the reaction time on the activity of the immobilized GOD is shown in Figure 5. The activity rapidly increased with increasing reaction time up to 8 h after which a tendency to level off was recognized. The pH above which the activity of the immobilized GOD decreased conformed with the pH level where a Cannizzaro reaction begins to occur together with aldol condensation (Margel and Rembaum, 1980). A Cannizzaro reaction leads to dismutation of the aldehyde group to hydroxyl and carboxyl groups. The appearance of those functional groups could cause a decrease of the hydrophobicity of the glutaraldehyde polymer, resulting in a reduced interaction with the hydrophobic surface of the microplate. At the pH where a Cannizzaro dismutation slowly proceeds (pH <10), the activity is possibly dependent only on the formation of the polymer by aldol condensation. From these results, the plate was subsequently activated with 10% glutaraldehyde solution (pH 10.0) for 20 h at 37 °C. Increased GOD concentration in the coupling had the effect to increase the activity of the immobilized GOD. The tendency to increase the activity leveled off after 360 μg of GOD in 200 μL of buffer. Thus, this concentration was chosen as the optimum one for the GOD solution for the immobilization in a subsequent experiment.

The time course of the oxidation reaction of glucose is shown in Figure 6. The absorbance, showing the formation of H_2O_2 by the reaction, increased to reach a plateau after ~30 min. If all of the glucose molecules added into the GOD-well were oxidized, the absorbance would give an absorbance value of ~2.5 in the case of 1.0 mM glucose because the solution that was transferred to the POD-well should contain the same concentration of H_2O_2 as the glucose concentration. However, both of the absorbance values of 1 and 0.2 mM glucose were approximately half that of the corresponding concentration of H_2O_2. There are three possibilities that could account for the observed results. The first possibility is that the GOD preparation could contain a catalase activity that decomposes H_2O_2 formed in the GOD-well. To check this, H_2O_2 solution (1 mM) was

added into the GOD-well and, after 30 min, an aliquot was transferred into the POD-well containing ABTS. Compared with the well without GOD, the absorbance was reduced to 90%, indicating that the GOD preparation involved a small but a certain activity of catalase. However, it is impossible to explain the smaller absorbance for glucose only by the catalase activity. The second possibility could be due to mutarotation of glucose. GOD can oxidize β-form of glucose. The α-form is much more slowly oxidized, so the mutarotation of α-form into β-form may be rate limiting (Kunst et al., 1984). The rate of the mutarotation generally depends on the kind of buffer used and the temperature. The use of high concentration of phosphate (for example 0.1 M) facilitates the reaction, and thus the α-form can be spontaneously converted into β-form as the latter is consumed (Narinesingh et al., 1991). In this experiment, 0.1 M phosphate buffer was used. The use of higher concentration of the buffer failed to increase the absorbance (data not shown). Therefore, it seems unlikely that the mutarotation is rate limiting. The third possibility is the consumption of dissolved oxygen accompanied by the oxidation of glucose. The concentration of dissolved oxygen in 0.1 M phosphate buffer at 22 °C is <300 μM (Wise and Naylor, 1985), meaning that this concentration corresponds to the maximum concentration of glucose to be oxidized. As shown in Figure 6, 1 mM glucose gave a much higher absorbance than 0.2 mM glucose, suggesting that the consumed oxygen was supplied from air. This could be due to the mixing of the microplate in an open condition to air at a speed of 500 rpm. As mentioned later, the calibration curve of glucose with the absorbance at each plateau showed linearity up to 1.5 mM. The D-glucose solution of lower concentration than dissolved oxygen concentration gave the absorbance along the calibration curve, so it is difficult to say that the supply of oxygen from air is rate limiting in the linear range. Although at the present stage the reason remains to be clarified, a further experiment using other oxidases might elucidate it. The absorbance reached at the plateau at $\sim$30 min, so this reaction time was chosen for a subsequent determination.

Under the conditions just described, the calibration curve for glucose was linear in the range 0.1−1.5 mM, with a correlation coefficient of 0.999 [$y = 1.10x + 0.08$; y, absorbance at 415 nm; x, glucose concentration (mM)]. The relative standard deviation was 2% for 1 mM glucose by six successive determinations with an identical well. When the plate was stored at 5 °C, the activity of the immobilized GOD was maintained for at least 120 days. The immobilized GOD on the plate was relatively stable and no significant decrease in the activity was observed for 30 successive determinations of 0.2 and 1.0 mM.

Determination of Glucose in Practical Sample. Based on the developed method with ABTS, any redox compound present in food samples may seriously affect the determination of glucose. In analyzing fruit juices, ascorbic acid is generally the most troublesome component that affects the redox reaction. There was no significant difference in the absorbance for each glucose concentration (0.2 and 1.0 mM) because up to 0.1 mM of ascorbic acid was added into each glucose solution. Above this concentration, however, the absorbance decreased with a slope of 1.6/1 mM ascorbic acid. Thus, ascorbic acid in a sample must be diluted to a concentration of <0.1 mM.

Table 1. Comparison of D-Glucose Concentration Obtained by the Present Immobilized Enzyme-Based Microtiter Plate Assay with That by the Conventional Enzymatic Method (F-Kit Method)[a]

sample	concentration, mM	
	present method	F-kit method
orange 1	122	113
orange 2	132	131
orange 3	116	116
orange 4	120	114
squeezed orange	124	135
apple 1	144	137
apple 2	140	134
grape	301	299
grapefruits	136	132

[a] Each sample was diluted 200- or 400-fold with 0.1 M phosphate buffer (pH 6.5); each value represents the mean of duplicate determinations.

The D-glucose concentrations in eight commercial fruit juices and beverages and one freshly squeezed orange juice were determined by the F-kit method (Table 1). Each sample was diluted 200−400-fold with buffer to within the linear range of glucose concentration as determined by the present microplate method. The ascorbic acid concentration in citrus fruit juices ranges from 1.7 to 2.8 mM (from 30 to 50 mg%; Tsumura et al., 1993; Casella et al., 1989). Apple and grape contained much lower ascorbic acid concentration than citrus fruits. When the sample was diluted 200-fold, the final concentration of ascorbic acid was reduced to <0.02 mM. At these low concentrations, ascorbic acid should have no effect on the determination of glucose in all fruit juices examined here. As expected, the glucose concentration obtained by the present method agreed well with that determined by the F-kit (Table 1). This result means that the developed microplate assay method can be applied to the determination of glucose in various fruit juices. The F-kit method is an enzymatic, spectrophotometric method for the determination of glucose based on the use of free enzymes and coenzyme. Compared with the F-kit method, the present method has a remarkable property that no exogenous addition of the enzyme into the assay solution is needed because the microtiter plate is equipped with the enzymes in the stable form. The immobilized POD and GOD on glutaraldehyde-activated microtiter plate showed a good reusability for a total of 30 repetitive uses. In the assay of glucose, therefore, only ABTS is required except the buffer. One gram of ABTS permits analysis of >5000 samples under optimum conditions, meaning that there could be substantial cost saving.

The combination of POD with oxidases other than GOD would permit construction of assay systems for other components. The microtiter plate is suitable for integrating many reaction vessels. When those vessels are designed for different components, it would be also possible to simultaneously determine multicomponents in a given sample with one microtiter plate.

LITERATURE CITED

Berkowitz, D. B.; Webert, D. W. The inactivation of horseradish peroxidase by a polystyrene surface. *J. Immunol. Methods* **1981**, *47*, 121−124.

Bilitewski, U. Enzyme electrodes for food analysis. In *Food Biosensor Analysis*; Wagner, G., Guilbault, G., Eds.; Dekker: New York, 1994; pp 31−61.

Casella, L.; Gullotti, M.; Marchesini, A.; Petrarulo, M. Rapid enzymatic method for vitamin C assay in fruits and vegetables using peroxidase. *J. Food Sci.* **1989**, *54*, 374−375.

Dobbins Place, J.; Schroeder, H. The fixation of anti-HBsAg on plastic surfaces. *J. Immunol. Methods* **1982**, *48*, 251−260.

Kunst, A.; Draeger, B.; Ziegenhorn, J. Colorimetric methods with glucose oxidase and peroxidase. In *Methods of Enzymatic Analysis*, 3rd ed., vol. VI; Bergmeyer, H. U., Ed.; VCH: Weinheim, 1984; pp 178−185.

Lee, M.-H.; Chen, R. L. C.; Matsumoto, K. Fluorometric biosensing of the total amino acid content and the glutamate content of green tea infusions using an automated multichannel flow system. *Biosci., Biotechnol., Biochem*. **1996**, *60*, 99−102.

Lopez-Fernandez, J. M.; Rios, A.; Valcarcel, M. Assessment of quality of flow injection methods used in food analysis. *Analyst* **1995**, *120*, 2393−2400.

Margel, S.; Rembaum, A. Synthesis and characterization of poly(glutaraldehyde). A potential reagent for protein immobilization and cell separation. *Macromolecules* **1980**, *13*, 19−24.

Matsumoto, K.; Matsubara, H.; Hamada, M.; Ukeda, H.; Osajima, Y. Simultaneous determination of glucose, ethanol and lactate in alcoholic beverages and serum by amperometric flow injection analysis with immobilized enzyme reactors. *J. Biotechnol*. **1990**, *14*, 115−126.

McCoy-Messer, J.; Bateman Jr., R. C. Instability of the ABTS/peroxidase reaction product in biological buffers. *BioTechnique* **1993**, *15*, 174−176.

Narinesingh, D.; Stoute, V. A.; David, G.; Ngo, T. T. Flow injection analysis of lactose using covalently immobilized β-galactosidase, mutarotase, and glucose oxidase/peroxidase on a 2-fluoro-1-methylpyridinium salt-activated fractogel support. *Anal. Biochem*. **1991**, *194*, 16−24.

Putter, J.; Becker, R. Peroxidase. In *Methods of Enzymatic Analysis*, 3rd ed., vol. III; Bergmeyer, H. U., Ed.; VCH: Weinheim, 1984; pp 286−293.

Rasmussen, K.-E.; Albrechtsen, J. Glutaraldehyde. The influence of pH, temperature, and buffering on polymerization rate. *Histochemistry* **1974**, *38*, 19−26.

Schwedt, G.; Stein, K. Immobilized enzymes as tools in food analysis. *Z. Lebensm. Unters. Forsch*. **1994**, *199*, 171−182.

Tsumura, F.; Ohsako, Y.; Haraguchi, Y.; Kumagai, H.; Sakurai, H.; Ishii, K. Rapid enzymatic assay for ascorbic acid in various foods using peroxidase. *J. Food Sci*. **1993**, *58*, 619−622.

Ukeda, H. Food analysis by biosensor-flow injection analytical methods. *Nippon Shokuhin Kagaku Kogaku Kaishi* **1995**, *42*, 849−858 (in Japanese).

Ukeda, H.; Ishii, T.; Sawamura, M.; Kusunose, H. Dynamic analysis of binding process of bovine serum albumin on glutaraldehyde-activated controlled pore glass. *Anal. Chim. Acta* **1995**, *308*, 261−268.

Welinder, K. G.; Mazza, G. Amino-acid sequence of heme-linked, histidine-containing peptides of five peroxidases from horseradish and turnip. *Eur. J. Biochem*. **1977**, *73*, 353−358.

Wilson, M. B.; Nakane, P. K. In *Immunofluorescence and Related Techniques*; Knapp, W., Holubar, H., Wick, G., Eds.; Elsevier: Amsterdam, 1978; 215 pp.

Wise, R. R.; Naylor, A. W. Calibration and use of a Clark-type oxygen electrode from 5 to 45 °C. *Anal. Biochem*. **1985**, *146*, 260−264.

Yoshioka, S.; Ukeda, H.; Matsumoto, K.; Osajima, Y. Simultaneous flow injection analysis of L-lactate and L-malate in wine based on the use of enzyme reactors. *Electroanalysis* **1992**, *4*, 545−548.

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