Journal of Health Science, **45**(3) 126—129 (1999)

Glucose Determination Using a Flow System with Enzyme Reactor and Application to Analysis of Glucose Content in Beverages

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(Received February 25, 1999; Accepted March 23, 1999)

Glucose was determined using an apparatus containing an enzyme reactor in a flow line. The enzymes used for glucose assay were hexokinase and glucose-6-phosphate dehydrogenase. NADH formed by enzymatic reactions was fluorometrically detected. The optimal concentrations of ATP and NAD^+ in the carrier were determined. Of the buffers examined for use as a carrier medium, triethanolamine buffer was found to be the most favorable and the optimal pH of this buffer was pH 7.0. Under these optimal conditions, the calibration curve for glucose was linear in the range of 2.0—200 μM ($r=0.999$). Detection limit was 1.7 μM ($S/N=3$). This method was applied to the analysis of glucose in several beverages. Glucose content determined by the present method agreed with that determined by a commercially available test-kit method. The methods for the preparation of immobilized enzymes were examined to retain the activities of enzymes.

Key words —— glucose, immobilized enzyme, flow injection analysis, beverage

INTRODUCTION

We previously reported[1] the determination of sucrose using immobilized enzymes in a flow system and applied it to the analysis of sucrose in beverages. Our interest then turned to the analysis of glucose which is another main component of a beverage. Glucose has been determined by enzyme systems such as mutarotase/glucose dehydrogenase and hexokinase (HK) /glucose-6-phosphate dehydrogenase (G6PD), where it is converted to NAD(P)H from $NAD(P)^+$ and the NAD(P)H formed was detectable. In the use of glucose oxidase, glucose is oxidized to form H_2O_2. Hydrogen peroxide formed is detected electrochemically[2,3] or by the measurement of chemiluminescence[4,5] emitted in the reaction of H_2O_2 with a chemiluminescent reagent. In a flow system with an enzyme reactor, NADH formed by enzymatic reactions can easily be detected spectrometrically or fluorometrically, in contrast to the H_2O_2 detection by chemiluminescence. Rarely has the determination of glucose by the NADH detection been reported using enzymes other than glucose oxidase in a flow system. Thus, the present study examined the determination of glucose by a flow system with a reactor containing immobilized HK and G6PD. Enzymatic reactions utilized are shown below.

$$\text{glucose} + \text{ATP} \xrightleftarrows{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NAD}^+ \xrightleftarrows{\text{G6PD}} \text{6-phosphogluconolactone} + \text{NADH}$$

In establishing this method, the optimal concentrations of ATP and NAD^+ in the carrier were determined for these condition. The buffer type for the carrier medium was explored to determine the best condition for the assay of glucose.

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MATERIALS AND METHODS

Materials —— G6PD from *Leuconostoc mesenteroides*, HK from bakers yeast, NAD^+, 25% aqueous solution of glutaraldehyde and aminopropyl glass (500 Å pore size, 200—400 mesh) were purchased from Sigma Chemical Co. (U.S.A.).

Enzyme Immobilization —— Enzymes were immobilized as described below. To aminopropyl glass beads (0.2 g) in 1.8 ml of 0.1 M phosphate buffer (pH

10.0) was added 0.2 ml of 25% aqueous solution of glutaraldehyde. The mixture was bubbled with N_2 gas for 1 h at room temperature. After the activated aminopropyl glass was filtered and washed with 50 ml water, 1.6 ml of 0.05 M phosphate buffer (pH 6.0), 20 units of G6PD and 20 units of HK were added. The mixture was stirred with a shaker for 20 h at 4℃. Enzyme-immobilized glass beads were filtered and washed with 50 ml of 0.05 M phosphate buffer (pH 6.0) and then with 50 ml of water. They were stored in 1.6 ml of 0.1 M triethanolamine buffer (pH 7.0) containing 2 mM ATP and 1.5 mM NAD^+ at 4℃. Fourteen percent and 10% of the activities of the enzymes initially applied were found for G6PD and HK, respectively. The enzyme-immobilized glass beads thus prepared were packed into a stainless steel column (4 cm, i.d. 2 mm).

Apparatus —— A schematic diagram of the apparatus used in the study is shown in Fig. 1. The enzyme reactor (ER) containing immobilized HK and G6PD was immersed in a water bath (WB) thermostated at 30℃. The carrier used was 0.1 M triethanolamine buffer (pH 7.0) containing 2 mM ATP, 1.5 mM NAD^+, and 10 mM $MgCl_2$, and this was delivered at a rate of 0.3 ml/min by a Hitachi L-6200 pump (P). NADH formed by the enzymatic reactions was fluorometrically detected (λ_{ex}, 340 nm; λ_{em}, 460 nm) by a Hitachi F-1000 spectrofluorometer (D) with a 12 μl flow cell. The peak area was obtained by a Hitachi D-2500 data processor (DP). The sample injection volume was 50 μl.

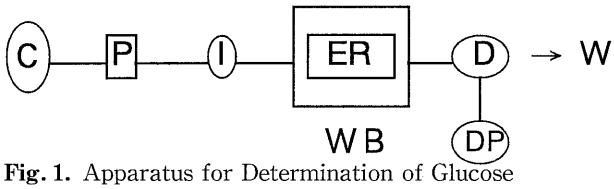

Fig. 1. Apparatus for Determination of Glucose

C, carrier reservoir; P, pump; I, sample injector; ER, enzyme reactor; D, spectrofluorometer; DP, data processor; WB, water bath; W, waste.

RESULTS AND DISCUSSION

Carrier solution contains ATP, NAD^+ and $MgCl_2$, and optimal concentrations of the first two substrates were studied. Fig. 2 shows the dependence of the peak area of NADH formed upon the concentration of ATP; concentrations of more than 1.5 mM afforded an almost constant peak area. Consequently, a concentration of 2 mM was applied in subsequent experiments. Fig. 3 shows the dependence of the peak area upon the concentration of NAD^+; the peak area reached almost maximum value at a concentration of 1.5 mM. Thus, this concentration was used in the subsequent experiments.

Table 1 lists the relative peak areas when various buffers (0.1 M, pH 7.5) were used as the carrier medium. Triethanolamine buffer gave the greatest peak area. Dependence of the peak area

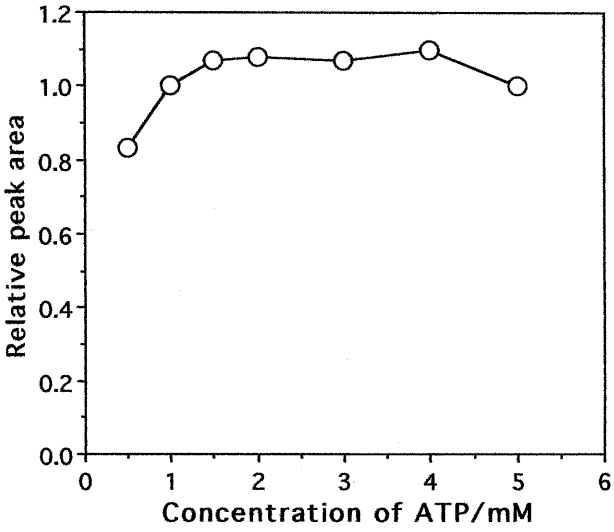

Fig. 2. Dependence of Peak Area upon ATP Concentration in the Carrier

Ordinate indicates the peak area relative to that at 1 mM ATP. Carrier used was 0.1 M Tris (pH 7.5) containing ATP, 1 mM NAD^+ and 10 mM $MgCl_2$. Flow rate of the carrier was 0.5 ml/min. Temperature of the water bath in which ER was immersed was 30℃. Concentration of glucose injected was 100 μM.

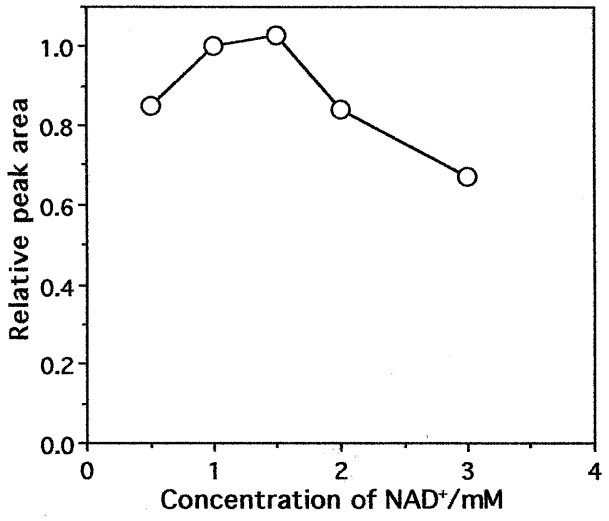

Fig. 3. Dependence of Peak Area upon NAD^+ Concentration in the Carrier

Ordinate indicates the peak area relative to that at 1 mM NAD^+. Carrier used was 0.1 M Tris (pH 7.5) containing NAD^+, 2 mM ATP and 10 mM $MgCl_2$. Other conditions were the same as those described in the legend of Fig. 2.

Table 1. Relative Peak Area due to NADH Using Various Buffers (0.1M, pH 7.5) as the Carrier Medium

Buffer	Tris	Phosphate	HEPES	PIPES	Triethanolamine
Relative peak area	1	0.38	0.74	0.83	1.56

Carrier contains 2 mM ATP, 1.5 mM NAD^+ and 10 mM $MgCl_2$. Other conditions were the same as described in the legend of Fig.2.

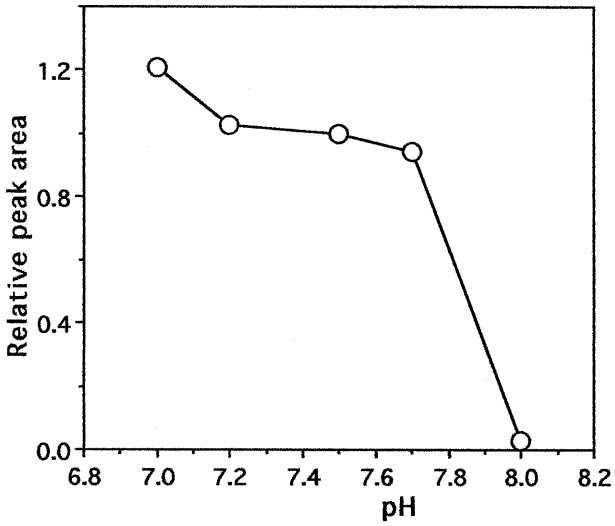

Fig. 4. Dependence of Peak Area upon pH of Carrier

Ordinate indicates the peak area relative to that at pH 7.5. Carrier used was 0.1 M triethanolamine buffer containing 2 mM ATP, 1.5 mM NAD^+ and 10 mM $MgCl_2$.

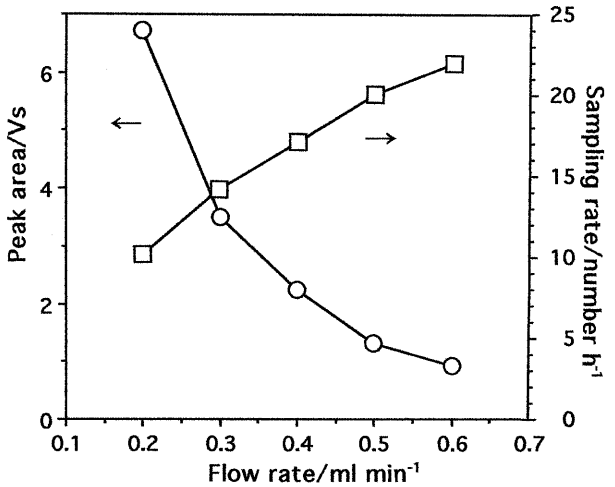

Fig. 5. Effect of the Flow Rate of Carrier upon Peak Area and Sampling Rate

Peak area is represented in the unit of Volt · second (Vs).

upon the pH of triethanolamine buffer as the carrier medium was shown by this area increasing with the decrease of pH value (Fig. 4). Thus, the triethanolamine buffer of pH 7.0 was adopted as a carrier medium.

Table 2. Glucose Content in Some Beverages (g/l)

	Proposed method	F-kit
Apple juice	26.2	26.5
Grapefruit juice	21.7	21.5
Tea	0.47	0.47
Tea + glucose[a)]	0.63	

Values are average of triplicate determinations. The samples for analysis were prepared by diluting original beverage with the carrier 2000 times for apple juice and grapefruit juice, and 50 times for tea. *a*) Theoretical value obtained from the added weight of glucose was 0.62 g/l.

Fig. 5 shows that the peak area decreased as flow rate of the carrier increased. However, the shape of the peak broadened and a long period of analysis was required at low flow rate. The sampling rate (number h^{-1}) calculated from the peak width at the base line is also shown in the figure. The flow rate of 0.3 ml/min was used in subsequent experiments as a compromise between the peak area and sampling rate.

The calibration curve under the above conditions was linear ($r=0.999$) in the glucose concentration range from 2.0 to 200 μM. The detection limit ($S/N=3$) was 1.7μM, and the relative standard deviation of the peak area at 10μM was 5.4% ($n=7$).

The glucose content in some beverages was determined by this method. Table 2 lists the results of analysis, compared with those obtained by a commercially available test kit (F-kit, Boehringer Mannheim), which showed good agreement. The glucose content in tea was considerably lower in comparison with those in other beverages. Glucose added sample was also analyzed, and again the content agreed with the calculated value (recovery was 107%).

The peak area for 10μM glucose was almost constant during the analysis of 50 samples including standard samples for calibration curves. However, one day following the analysis, a distinct decrease in peak area was observed owing to the loss of activities of the immobilized enzymes. Thus, other methods of preparation of the immobilized enzymes than that described in "Materials and Methods" were examined. One technique includes the addition of bovine serum albumin (BSA) after the reaction of enzymes with activated aminopropyl glass. Another includes the addition of sodium cyanoborohydride (SCB) to reduce C=N bond in the chain between enzyme and glass. The results are shown in Fig.

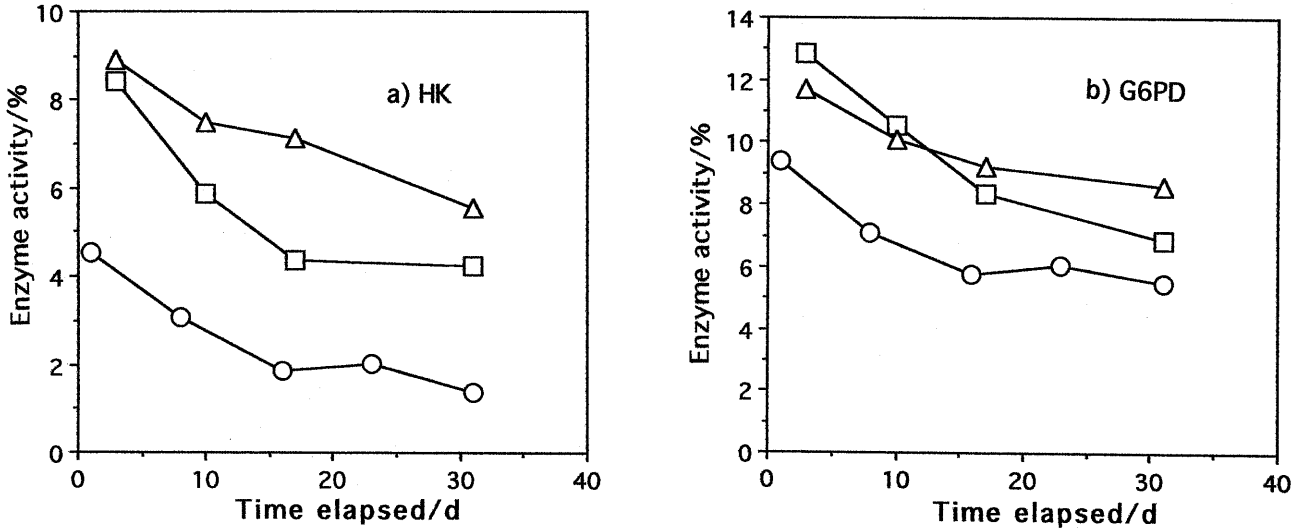

Fig. 6. Time Course of the Activities of Immobilized Enzymes

—□—, no addition ; —△—, BSA addition ; —○—, SCB addition. Ordinate indicates the ratio (%) of the activity of immobilized enzyme (units) to that of added soluble enzyme (units). The immobilized enzymes without addition were prepared according to the method described in "Materials and Methods." The immobilized enzymes added with BSA were prepared in a similar manner to those without addition except that after the reaction of two enzymes with glutaraldehyde activated glass beads for 20 h, 1% (w/v) of BSA was added to the reaction mixture and it was shaken for 4 h. The immobilized enzymes added with SCB were prepared by adding 1.6% (w/v) of SCB instead of BSA.

6a and Fig. 6b, indicating that the BSA addition method was superior to others in retaining the activities of both HK and G6PD.

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