Determination of Isocitrate Using Immobilized Isocitrate Dehydrogenase in a Flow System and its Application to Analyze the Total Isocitrate Content of Beverages

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The quantity of isocitrate was determined using an apparatus containing a reactor with immobilized isocitrate dehydrogenase in a flow line. NADH formed by an enzymatic reaction was fluorometrically detected. The optimal concentration of NAD^+ in the carrier was determined. The maximum peak areas due to NADH were observed at pH 8.0 when the pH of the carrier consisting of Tris buffer ranged from 6.0 to 8.6. Various buffer types were also examined as carrier mediums at pH 8.0. In contrast to 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and triethanolamine buffers which afforded comparable peak areas with that of Tris buffer, phosphate buffer showed a reduced peak area. This peak area decreased with an increase in the pH of the phosphate buffer from 6.5 to 8.5, suggesting the inhibitory effect of phosphate dianions upon the binding of adenosine-5′-monophosphate (AMP) to the binding site of the enzyme. When the carrier composed of Tris buffer (0.1 M, pH 8.0) was used, the calibration curve for isocitrate was linear in the range of 0.1–50 μM (r = 1.000). The detection limit (S/N = 3) was 0.07 μM. Relative standard deviations of the peak area at 1 and 10 μM were 4.0% (n = 7) and 2.8% (n = 7), respectively. Thirty samples of isocitrate (2 μM) were analyzed for 1 hr. This method was applied to the analysis of total isocitrate in several beverages. The recovery tests for the isocitrate added to samples indicatied the reliability of the present method.

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Key words —— isocitrate, isocitrate dehydrogenase, immobilized enzyme, flow injection analysis, beverage

INTRODUCTION

Immobilized enzymes have been widely used as enzyme reactors in flow systems or as enzyme electrodes. Immobilized enzymes have also been used in reactors to determine the components of drinks: glucose,[1] sucrose,[2] acetaldehyde,[3] both glucose and sucrose,[4] both D- and L-lactic acid,[5] and both acetaldehyde and ethanol.[6]

In the present work, the determination of isocitrate was studied using immobilized NAD^+-dependent isocitrate dehydrogenase (ICDH) in a flow system. The determination of isocitrate has been carried out particularly for citrus fruits and in the products generated from them, because its concentration has become an important indicator of the level of adulteration of citrus beverages.[7] Thus, the present method was used to try to determine the total isocitrate contents of various beverages. The enzymatic reaction utilized is shown below; NADH formed by this reaction was detected fluorometrically.

$$\text{isocitrate} + NAD^+ \xrightarrow{\text{ICDH}} \text{2-oxoglutarate} + CO_2 + NADH$$

MATERIALS AND METHODS

Materials —— NAD^+-dependent isocitrate dehydrogenase (ICDH, EC 1.1.1.41) from yeast, NAD^+ and adenosine-5′-monophosphate (AMP) were purchased from the Oriental Yeast Co., Ltd. (Japan), the 25% aqueous solution of glutaraldehyde was from the Sigma Chemical Co., Ltd. (U.S.A.), isocitric acid trisodium salt was from ICN Biomedicals, Inc. (U.S.A.), and aminopropyl glass (500 Å pore size, 200–400 mesh), was from CPG, Inc. (U.S.A.).

Enzyme Immobilization —— ICDH was immobilized as described below. To aminopropyl glass beads (0.4 g) in 3.6 ml of 0.1 M phosphate buffer (pH 10.0) was added 0.4 ml of a 25% aqueous solution of glutaraldehyde. The mixture was bubbled with N_2 gas for 1 hr at room temperature. After the activated aminopropyl glass was filtered and washed with 100 ml water, 3.2 ml of 0.05 M phosphate buffer (pH 6.0) and 100 units of ICDH were added.

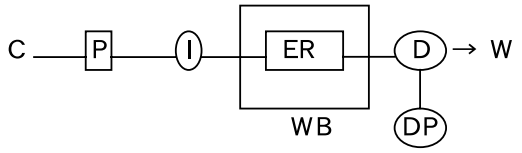

Fig. 1. Apparatus for the Determination of Isocitrate

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

The mixture was stirred with a shaker for 20 hr at 4°C, and it was shaken for a further 4 hr after bovine serum albumin (BSA) was added to it to become 1%(w/v). Enzyme-immobilized glass beads were filtered and washed with 100 ml of 0.05 M phosphate buffer (pH 6.0) and then with 100 ml of water. They were stored in 3.2 ml of 0.05 M phosphate buffer (pH 6.0). Ten point three percent of the activity of the ICDH initially applied was found in the immobilized enzyme. The enzyme-immobilized glass beads thus prepared were packed into a stainless steel column (5 cm, i.d. 2 mm).

Apparatus —— A schematic diagram of the apparatus used in the present study is shown in Fig. 1. The enzyme reactor (ER) containing immobilized ICDH was immersed in a water bath (WB) thermostated at 30°C. The carrier used was 0.1 M Tris buffer (pH 8.0) containing 1.0 mM NAD^+, 0.1 mM AMP and 5 mM $MgCl_2$, and this was delivered at a rate of 0.4 ml min^{-1} by a Shimadzu LC-10AD pump (P). NADH formed by the enzymatic reaction was fluorometrically detected (λ_{ex}, 340 nm; λ_{em}, 460 nm) by a Hitachi F-1050 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.

The Preparation of the Samples for the Determination of Total Isocitrate[7] —— This preparation method is that for the determination of total isocitrate including free acid, ester and lactone. Twenty-five ml of a real sample was accurately taken into a flask and adjusted the pH in the range from 10 to 11 with 2 mol/l NaOH aqueous solution. After the addition of 0.01 ml of 30% hydrogen peroxide to this solution, it was heated with boiling water for 20 min. The pH of this solution was periodically checked and adjusted the pH in the range from 10 to 11 with 2 mol/l NaOH aqueous solution. After cooling to room temperature, the pH of the solution was adjusted in the range from 6.9 to 7.2 with 1 mol/l HCl. The solution was transferred to a 50 ml volumetric flask. After it was stirred with 0.2 g bentonite for 1 min, water was added up to the marked line of the flask. The filtrate of the solution was used for the analysis. Thus, the sample for the determination of the total isocitrate was the doubly diluted solution of the original sample.

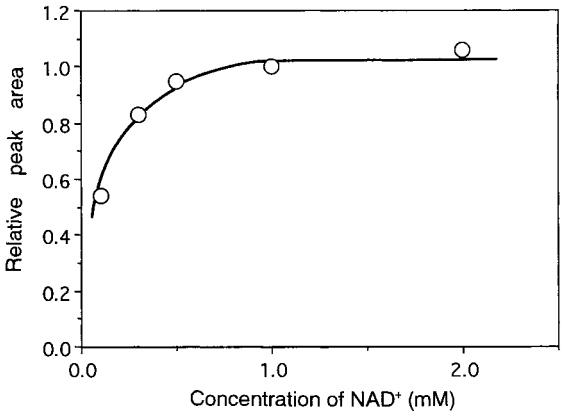

Fig. 2. Dependence of the Peak Area upon the NAD^+ Concentration of the Carrier

Ordinate indicates the peak area relative to that at 1.0 mM NAD^+. Values were obtained from the averages of triplicate determinations. Carrier used was 0.1 M Tris buffer (pH 8.0) containing NAD^+, 5 mM $MgCl_2$ and 0.5 mM AMP. Flow rate of the carrier was 0.4 ml min^{-1}. Temperature of the water bath in which ER was immersed was 30°C. The concentration of isocitrate injected was 50 μM.

RESULTS AND DISCUSSION

The carrier solution contained AMP, magnesium ions ($MgCl_2$), and NAD^+. AMP is needed[8] as the activator of lCDH. The effect of the concentration of NAD^+ in the carrier upon the peak area due to NADH was examined. As shown in Fig. 2, NAD^+ concentrations of more than 1.0 mM afforded an almost constant peak area. Consequently, a concentration of 1.0 mM was used in the subsequent experiments.

Figure 3 shows the dependence of the peak area upon the pH of Tris buffer in the pH range from 6.0 to 8.6. The maximum peak area was obtained at pH 8.0. Various buffer types were also examined as carrier mediums. Table 1 shows the effect of each buffer type (0.1 M, pH 8.0) as a carrier medium upon the peak area of NADH. All buffers other than phosphate and pyrophosphate buffer afforded large peak areas comparable with that of Tris buffer. Figure 3 also shows the dependence of the peak area upon the pH of the phosphate buffer. The optimum pH was 6.5, and the peak area decreased with an in-

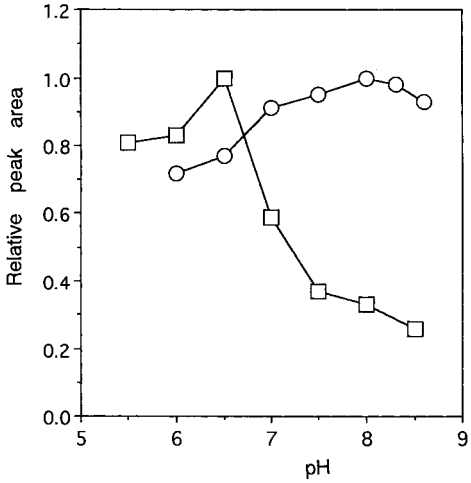

Fig. 3. Dependence of the Peak Area upon the Carrier pH

—○—, carrier composed of Tris buffer; —□—, carrier composed of phosphate buffer. Ordinate indicates the peak area relative to that at pH 8.0 for Tris buffer and that at pH 6.5 for phosphate buffer. Values were obtained from the averages of triplicate determinations. Carrier used contained 1.0 mM NAD^+, 5 mM $MgCl_2$ and 0.5 mM AMP. Other conditions were the same as those described in the legend of Fig. 2.

Table 1. The Effects of Different Buffer Types (0.1 M, pH 8.0) as Carrier Mediums upon the Peak Area due to NADH

Buffer	Relative peak area
Tris	1.00
Phosphate	0.33
Pyrophosphate	0.00
HEPES	1.04
PIPES	0.87
Triethanolamine	0.90

Values are the peak areas relative to that obtained in the case of Tris buffer and they are the averages of triplicate determinations. Carriers contain 1.0 mM NAD^+, 5 mM $MgCl_2$ and 0.5 mM AMP. Other conditions were the same as described in the legend of Fig. 2.

crease in pH of the carrier in the range from 6.5 to 8.5. Of the components of phosphate buffer, the concentration of phosphate dianions increases as the buffer becomes alkaline in the pH range mentioned above. Thus, in this pH range, the activator AMP have difficulty binding to the binding site of the enzyme through the competition with the phosphate dianion. Pyrophosphate seems to completely inhibit the binding of AMP. The difference in the pH dependence of the peak areas observed with the Tris and phosphate buffers in the case of immobilized enzyme was in contrast to that in the case of soluble ICDH obtained by Kornberg *et al.*[8]; the rates of the activities obtained with phosphate and Tris buffers at given pH values (from 6.5 to 8.5) were in agreement.

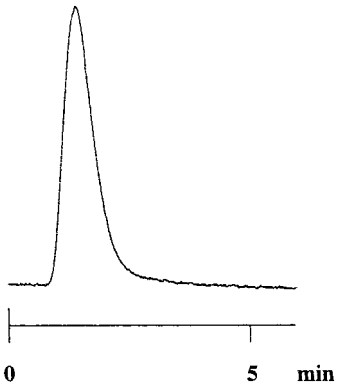

Fig. 4. Peak due to Isocitrate

The peak indicated was that of 5 μM isocitrate observed under the conditions described in the legend of Fig. 3 using Tris buffer.

Table 2. Total Isocitrate Contents of Some Beverages (mM)

Grapefruit juice-1	3.92 ± 0.16
Grapefruit juice-2	9.42 ± 0.14
Orange juice-1	7.58 ± 0.10
Orange juice-2	5.86 ± 0.04
Lemon water	0.48 ± 0.06
Apple juice	1.72 ± 0.02
Acerola drink	0.69 ± 0.02

Values are the averages of triplicate determinations. The total isocitrate values in this Table were obtained by doubling those for the samples prepared as described in the Experimental section.

Tris buffer was used as the carrier medium for the following experiments and the pH selected for the carrier was 8.0. The peak due to isocitrate was observed at 1.3 min under the experimental conditions, as shown in Fig. 4. The calibration curve obtained was linear (r = 1.000) in the isocitrate concentration range from 0.1 to 50 μM. The detection limit (S/N = 3) was 0.07 μM. The relative standard deviation of the peak area was 4.0% (n = 7) and 2.8% (n = 7) at 1 and 10 μM, respectively. These values seems to be small, compared with that found in the gas chromatographic study,[9] 7.5% in the concentration 1.2×10^{-3}–9.0×10^{-3} g isocitrate per 100 g. The 30 times analysis of the isocitrate sample (2 μM) could be accurately carried out in one hour by the present method. And this analysis rate is in contrast to that in the analysis using soluble enzyme[7] in which it takes more than 15 min to analyze a single sample. The sample of citrate showed no peak.

The present method was applied to the analysis of the total isocitrate contents of various beverages.

Table 3. Recovery of Isocitrate Added to Each Isocitrate Sample

	Added isocitrate (mM)	Observed isocitrate (mM)	Recovery (%)
Orange juice-2	0	2.93	
	1.50	4.47	103
	3.00	6.20	109
	6.00	9.22	105
Apple juice	0	0.86	
	0.50	1.44	116
	1.00	1.92	106
	2.00	2.92	103

The samples used for this test were those prepared as described in the Experimental section.

Table 2 lists the results of this analysis, showing that a large quantity of isocitrate was contained in citrus fruit juices. Recovery tests for the added isocitrate to the samples were also undertaken (Table 3), indicating the reliability of this method.

The peak area for 50 μM isocitrate was almost constant during the analysis of 200 samples, including standard samples for a calibration curve. After storage of the enzyme reactor for 9 months at 4°C in the carrier (pH 8.0) composed of Tris buffer, the activity of the enzyme in the reactor dropped to 95% of its initial activity. Thus, the immobilized isocitrate dehydrogenase is very stable compared with other immobilized enzymes.[1,3)]

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