Determination of D-Malate Using Immobilized D-Malate Dehydrogenase in a Flow System and its Application to Analyze the D-Malate Content of Beverages

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The quantity of D-malate was determined using apparatus comprised of a reactor with immobilized D-malate dehydrogenase (D-MDH) 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 piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer ranged from 7.0 to 8.5. Various buffer types were also examined as carrier media at pH 8.0, and PIPES buffer showed the maximum peak area. When the carrier composed of PIPES buffer (0.1 M, pH 8.0) containing 1 mM NAD^+ and 5 mM $MgCl_2$ was used, the calibration curve for D-malate was linear in the range of 0.02–50 μM (r = 1.000). The detection limit (S/N = 3) was 0.01 μM. Relative standard deviations of the peak area at 1 μM and 10 μM were 1.6% (n = 7) and 0.48% (n = 7), respectively. This method was applied to the analysis of D-malate in several beverages, and the recovery test of the added D-malate to samples was also carried out to afford good results.

Key words —— D-malate, D-malate dehydrogenase, immobilized enzyme, flow injection analysis, beverage

INTRODUCTION

Of two isomers of malic acid, L-malic acid occurs naturally, whereas D-malic acid is only found in the metabolism of microorganisms.[1] The racemate of malic acid can be produced chemically, so if malic acid is used as an acidulant, the beverage may contain D-malic acid as well as L-malic acid. The separation of D- and L-malic acid has been carried out by high performance liquid chromatography (HPLC)[2–6] and was applied to determine enantiomers in fruits juices.[4] We have utilized the flow systems containing immobilized enzyme for the determination of components of foods.[7–10] Compared with HPLC method, the time required for analysis by this method is short and many samples can be analyzed per time. Thus, the flow system containing immobilized D-malate dehydrogenase (D-MDH) was studied for the quantification of D-malate and applied to the determination of the content of D-malate in various beverages, testing the addition of the racemate of malic acid.

The enzymatic reaction[1] is shown below. In this reaction, decarboxylating D-MDH (E.C.1.1.1.83) is utilized. NADH formed by the reaction was detected fluorometrically.

$$\text{D-malate} + NAD^+ \xrightarrow{\text{D-MDH}} (\text{oxaloacetate}) \longrightarrow \text{pyruvate} + CO_2 + NADH + H^+$$

MATERIALS AND METHODS

Materials —— D-MDH was purchased from Roche-Diagnostics GmbH (Basel, Switzerland), NAD^+ was from the Oriental Yeast Co., Ltd. (Tokyo, Japan), D-malic acid and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) were from Wako Pure Chemicals Industries Ltd. (Osaka, Japan), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) were from Dojindo Laboratories (Kumamoto, Japan), the 25% aqueous solution of glutaraldehyde was from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.), and aminopropyl glass (500 Å pore size, 200–400 mesh) was from CPG, Inc. (Lincoln Park, NJ, U.S.A.).

Enzyme-Immobilization —— D-MDH were immobilized[7,8] 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 21 units of D-MDH were added. The mixture was stirred with a shaker

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for 20 hr at 4°C, and shaken for a further 4 hr after BSA was added to 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). Eleven percent of the activity of the initially applied enzyme was found in the immobilized enzyme. 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 this study is shown in Fig. 1. The enzyme reactor (ER) containing immobilized enzymes was immersed in a water bath (WB) at 30°C. The carrier used was 0.1 M PIPES buffer (pH 8.0) containing 1 mM NAD^+ 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.

Preparation of the Samples —— Immobilized D-MDH reacts slowly with tartaric acid, so the preparation of the samples for measurement includes the removal[11] of the effect of tartaric acid. Solid calcium hydroxide (50 mg) and ethanol (2 ml) were added to 10 ml of beverage. After the mixture was stirred for 2 min, its pH was adjusted to 7–8 with 2 M hydrochloric acid or 2 M sodium hydroxide. It was then quantitatively transferred to a 20 ml volumetric flask, filled with water to the marked line and mixed. The mixture was then filtered with filter paper. The filtrate was 50 times diluted with PIPES buffer (0.1 M, pH 8.0). This solution, 100 times diluted, of original beverage was used for analysis. In the case of grape juice, the filtrate was decolorized prior to dilution with PIPES buffer, adding 0.6 g of activated charcoal to the whole filtrate, stirring for 2 min and filtered.

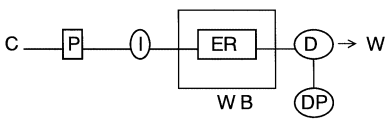

Fig. 1. Apparatus for the Determination of D-Malate

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

RESULTS AND DISCUSSION

The dependence of the NAD^+ concentration in the carrier upon the peak area due to NADH was examined. As shown in Fig. 2, NAD^+ concentrations of more than 1 mM afforded an almost constant peak area. Consequently, a concentration of 1 mM was used in subsequent experiments.

The dependence of the peak area upon the pH of the carrier comprised of PIPES buffer was investigated in the pH range from 7.0 to 8.5. Although the peak area did not show a notable difference in this pH range, the maximum peak area was obtained at pH 8.0. Various buffer types (0.1 M, pH 8.0) were also examined as carrier media. The order of the relative peak area due to NADH using various buffer types was PIPES (1.00) > HEPES (0.79) > Triethanolamine (0.58) > phosphate (0.35) > Tris (0.06). Thus, PIPES afforded the maximum peak area, whereas Tris buffer showed a considerably smaller peak area. PIPES buffer (0.1 M, pH 8.0) was used as the carrier medium for the following experiments.

The peak due to D-malate was observed at 1.2 min under the experimental conditions, as shown in Fig. 3, while even 50 μM of L-malate did not give a clear peak. The calibration curve obtained was linear (r = 1.000) in the D-malate concentration range from 0.02 to 50 μM. The detection limit (S/N = 3) was 0.01 μM. The relative standard deviation of the peak area was 1.6% (n = 7) and 0.48% (n = 7) at 1 μM and 10 μM, respectively. Tartaric acid

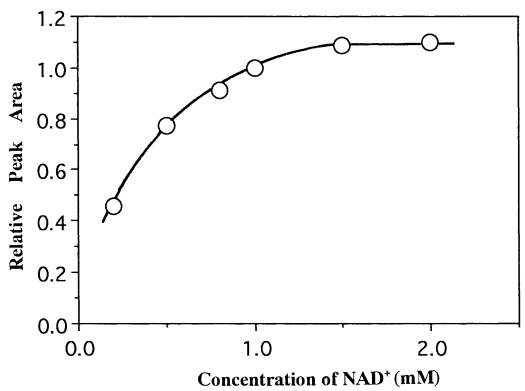

Fig. 2. Dependence of the Peak Area upon the NAD^+ Concentration of the Carrier

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

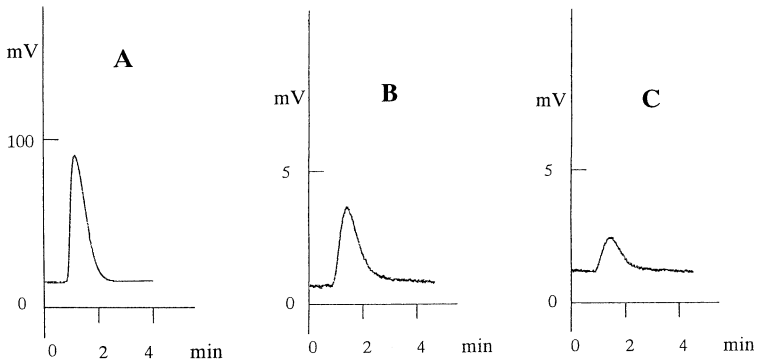

Fig. 3. Peaks of D-Malate and Real Samples

(A) 5 μM D-Malate, (B) Grape juice sample with PIPES buffer containing 1 mM NAD^+ and 5 mM $MgCl_2$ as carrier, (C) Grape juice sample with PIPES buffer as the carrier.

Table 1. D-Malate Contents of Beverages (μM)

Sample	Content
Apple juice-1	25 ± 1
Apple juice-2	27 ± 4
Lemon juice	61 ± 2
Orange Mikan juice	55 ± 5
Grape juice	40 ± 1

Values are the averages of triplicate determinations.

Table 2. Recovery of D-Malate Added to Beverages

Sample	Added D-Malate (μM)	Observed D-Malate (μM)	Recovery (%)
Apple juice-2	0	27	
	50	77	100
	100	123	96
	200	209	91
Grape juice	0	40	
	50	90	100
	100	129	89
	200	215	88

gave a small peak, and the slope of the calibration curve for tartaric acid was less than 1/500 of that for D-malate. Tsukatani and Matsumoto studied[12] the sequential fluoromeric quantification of malic acid enantiomers by a flow-injection system using immobilized-enzyme. The lower limit of detection of D-malate in their study (1 μM) is larger than that in the present study (0.02 μM). This may be mainly due the difference of the flow rate of the carrier, being 1.5 ml/min and 0.4 ml/min for their and for the present study, respectively.

The present method was applied to analyze the D-malate content of various beverages. The original sample seemed to involve a peak due to substances other than D-malate, so the peak area for D-malate was calculated by subtracting the peak area observed with the use of PIPES buffer (0.1 M, pH 8.0) as the carrier, from that observed with the use of PIPES buffer (0.1 M, pH 8.0) containing 1 mM NAD^+ and 5 mM $MgCl_2$ as the carrier. Figure 3 also shows the peaks of grape juice for use of the carrier with and without NAD^+ and $MgCl_2$, respectively. Table 1 lists the results of this analysis. The D-malate contents obtained by the present method were comparable with those[13] obtained when a soluble enzyme was used. The recovery test of the added D-malate was also carried out with respect to apple juice-2 and grape juice, affording good results, as shown in Table 2.

The peak area for 10 μM D-malate decreased to 82% of that initially detected after the analysis of 100 samples including standard samples for a calibration curve. After storage of the enzyme reactor for 6 months at 4°C in the carrier (pH 8.0) composed of PIPES buffer containing 1 mM NAD^+ and 5 mM $MgCl_2$, the peak area of 10 μM D-malate dropped to 15% of that before storage. Thus, the immobilized enzyme used in the present study is less stable than previously used immobilized enzymes.[9,10]

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