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

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The quantity of L-malate was determined using apparatus comprised of a reactor with immobilized malate dehydrogenase (MDH) and aspartate aminotransferase (AST) in a flow line. NADH formed by an enzymatic reaction was fluorometrically detected. The optimal concentration of NAD^+ in the carrier containing 0.1 M glutamate 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 7.0 to 8.5. Various buffer types were also examined as carrier media at pH 8.0 and Tris buffer showed the maximum peak area. When the carrier composed of Tris buffer (0.1 M, pH 8.0) was used, the calibration curve for malate was linear in the range of 0.05–50 μM (r = 1.000). The detection limit (S/N = 3) was 0.03 μM. Relative standard deviations of the peak area at 1 μM and 10 μM were 1.5% (n = 7) and 0.36% (n = 7), respectively. Thirty samples of malate (10 μM) were analyzed for 1 hr. This method was applied to the analysis of malate in several beverages, and malate content determined by this method agreed with that determined by a commercially available test-kit method.

Key words —— L-malate, malate dehydrogenase, aspartate aminotransferase, immobilized enzyme, flow injection analysis, beverage

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INTRODUCTION

Immobilized enzymes have been widely used as enzyme reactors in flow systems or as enzyme electrodes. The application of immobilized enzymes in reactors includes the determination of drinks components: glucose,[1] sucrose,[2] acetaldehyde,[3] isocitrate,[4] both glucose and sucrose,[5] both D- and L-lactic acid,[6] both acetaldehyde and ethanol.[7]

In this work, the determination of L-malate was studied using a reactor containing immobilized malate dehydrogenase (MDH) and aspartate aminotransferase (AST) in a flow system. L-malate is an intermediate of the citric acid cycle and is contained in many fruits. To evaluate the authenticity of fruit juices, the determination of specific acids, isocitrate and L-malate, which are present in measurable amounts only in genuine juices, was recommended.[8–11] Thus, this method was applied to determine the content of L-malate in various beverages.

The enzymatic reactions[12] are shown below. The equilibrium of the reaction (1) catalyzed by MDH is in favor of L-malate formation. The reaction (2) is utilized to remove oxaloacetate formed in the reaction (1), shifting the equilibrium of the reaction (1) to NADH formation. Thus, to achieve this shift, glutamate of high concentration (0.1 M) is contained in the carrier solution as well as NAD^+. NADH formed by the reaction was detected fluorometrically.

$$\text{L-malate} + NAD^+ \xrightarrow{\text{MDH}} \text{oxaloacetate} + NADH + H^+ \quad (1)$$

$$\text{oxaloacetate} + \text{L-glutamate} \xrightarrow{\text{AST}} \text{L-aspartate} + \text{2-oxoglutarate} \quad (2)$$

MATERIALS AND METHODS

Materials —— MDH (EC 1.1.1.37) from yeast, and NAD^+ were purchased from the Oriental Yeast Co., Ltd. (Tokyo, Japan), AST (EC 2.6.1.1) from pig heart, L-malic acid, L-glutamic acid and polyvinylpyrrolidone was from Wako Pure Chemicals Industries Ltd. (Osaka, 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.). F-kit is a test kit from Roche Diagnostics GmbH.

Enzyme Immobilization —— MDH and AST were co-immobilized[5,6] 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) ,100 units of MDH and 410 units of AST were added. The mixture was stirred with a shaker for 20 hr at 4°C, and shaken for a further 4 hr after BSA (Bovine Serum Albumin) 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). Six and twenty-six percent of the activity of the initially applied enzymes were found for MDH and AST, respectively, in immobilized enzymes. The enzyme-immobilized glass beads thus prepared were packed into a stainless steel column (8 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 Tris buffer (pH 8.0) containing 1.0 mM NAD^+ and 0.1 M glutamate, and this was delivered at a rate of 0.4 ml min^{-1} by a Shimadzu (Kyoto, Japan) LC-10AD pump (P). NADH formed by the enzymatic reaction was fluorometrically detected (λ_{ex}, 340 nm; λ_{em}, 460 nm) by a Hitachi (Tokyo, Japan) 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 —— Orange juice and grapefruit juice were centrifuged for 30 min at 4000 rpm and filtered with filter paper prior to decoloration. Decoloration was performed for all samples as below. To 10 ml of a sample, 0.1 grams of polyvinylpyrolidone was added and stirred for 1 min and filtered with filter paper. It was then diluted with the carrier solution for analysis.

Determination of L-malate by F-kit —— Three minutes after the mixing of NAD^+, AST and a sample containing L-malate in the glycylglycine buffer (pH 10.0), the absorbance of the mixture was measured (E_1). Then MDH was added to the mixture, and the absorbance (E_2) of this mixture was also measured after the completion of the reaction. The values of E_2 and E_1 were respectively calibrated with those by a blank test. The content of L-malate in the sample was calculated from the difference E_2 minus E_1 calibrated.

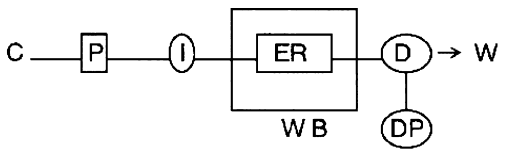

Fig. 1. Apparatus for the Determination of Malate

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 peak area due to NADH upon the NAD^+ concentration in the carrier 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 7.0 to 8.5. The maximum peak area was obtained at pH 8.0. Various buffer types were also examined as carrier media. Table 1 shows the effect of each buffer

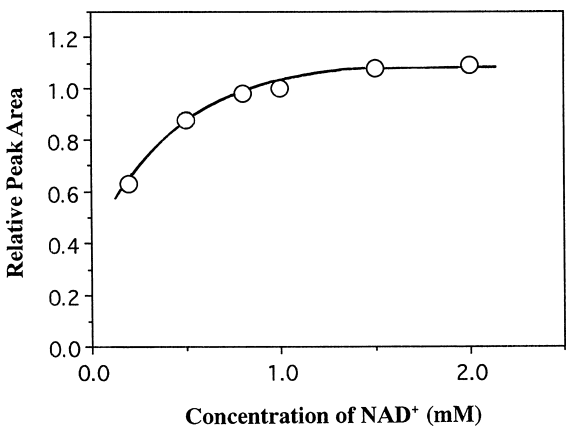

Fig. 2. Dependence of the Peak Area upon the NAD^+ Concentration of the Carrier

Ordinate indicates the peak area relative to that at 1.0 mM NAD^+. Values were obtained from the averages of triplicate determinations. Carrier was 0.1 M Tris buffer (pH 8.0) containing NAD^+ and 0.1 M glutamate. 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 malate injected was 50 μM.

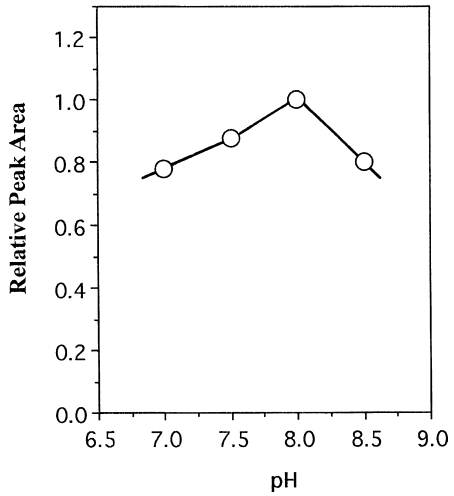

Fig. 3. Dependence of the Peak Area upon the Carrier pH

Ordinate indicates the peak area relative to that at pH 8.0. Values were obtained from the averages of triplicate determinations. Carrier used contained 1.0 mM NAD^+ and 0.1 M glutamate. Other conditions were the same as those described in the legend of Fig. 2.

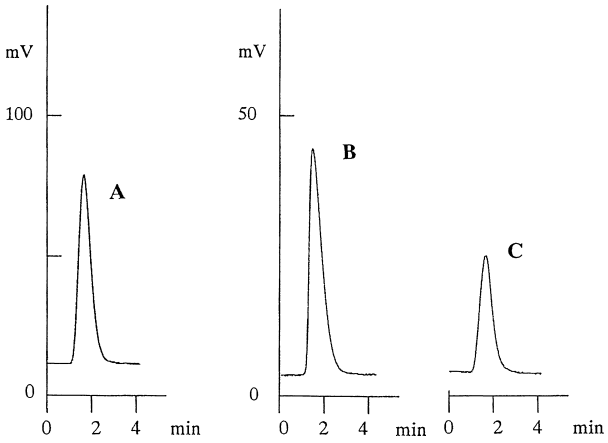

Fig. 4. Peak due to Malate

These peaks were observed under the conditions described in the legend of Fig. 3. (A) 5 μM Malate. (B) Apple juice sample 10000-fold diluted with the carrier. (C) Grape juice sample 10000-fold diluted with the carrier.

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

Buffer	Relative peak area
Tris	1.00
Phosphate	none
HEPES	0.92
Triethanolamine	0.74

Values are the peak areas relative to that obtained with Tris buffer, and are the averages of triplicate determinations. Carriers contained 1.0 mM NAD^+ and 0.1 M glutamate. Other conditions were as described in the legend of Fig. 2.

Table 2. Malate Contents of Beverages (mM)

	This method	F-kit method
Apple juice	29.8 ± 0.7	30.3
Grape juice	15.0 ± 0.03	17.9
Lemon juice	21.2 ± 0.1	22.8
Orange juice	9.86 ± 0.06	9.68
Grapefruit juice	3.47 ± 0.05	3.35
Acerola drink	1.73 ± 0.03	—[a)]

Values by this method are the averages of quintuplicate determinations. *a*) Accurate measurement could not be made due to the continuous increment of the absorbance of the mixture when soluble AST was mixed with a sample solution prior to the addition of soluble MDH.

type (0.1 M, pH 8.0) as a carrier medium upon the peak area of NADH. Of the buffers, Tris afforded the maximum peak area, whereas phosphate buffer showed no peak.

Tris buffer (0.1 M, pH 8.0) was used as the carrier medium for the following experiments. The peak due to malate was observed at 1.5 min under the experimental conditions, as shown in Fig. 4. The calibration curve obtained was linear (r = 1.000) in the malate concentration range from 0.05 to 50 μM. The detection limit (S/N = 3) was 0.03 μM. The relative standard deviation of the peak area was 1.5% (n = 7) and 0.36% (n = 7) at 1 μM and 10 μM, respectively. These values seem small compared with that found in the gas chromatographic study,[8)] 4.2% in the concentration 4.2×10^{-3}–3.5×10^{-2} g malate per 100 g. The 30 analyses of the malate sample (10 μM) could be accurately carried out in 1 hr by this method. This analysis rate is in contrast to that by the method using soluble enzyme,[12)] in which it takes more than 5 min to analyze a single sample using complicated procedures.

This method was applied to analyze the malate content of various beverages. In Fig. 4 were also shown the peaks due to malate in real samples. Table 2 lists the results of this analysis, compared with those obtained by a commercially available test kit (F-kit), which showed good agreement except for grape juice. The peak area for 25 μM malate decreased to 90% of that initially detected after the analysis of 130 samples including standard samples for a calibration curve. After storage of the enzyme reactor for 6 months at 4°C in a carrier (pH 8.0) composed of Tris buffer, the peak area of 25 μM malate dropped to 91% of that before storage. Thus, the immobilized enzymes used in these assays are relatively stable compared with other immobilized enzymes.[1,3)]

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