Improved Enzymatic Method for Determination of Lactate in Alcoholic Beverages

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A rapid enzymatic method for determination of lactate in alcoholic beverage was developed. Sodium bicarbonate-sodium hydroxide buffer was the most suitable for the determination of lactate among eight kinds of buffers. Suitable conditions for the LDH reaction were as follows: concentration and pH of buffer, 400 mM and 10.0; concentration of NAD, 2 mM; reaction temperature, 25°C; reaction time, 2 min for L-lactate, 15 min for D-lactate. The present method gave a good recovery of lactate at concentrations of more than 1 mg/l in alcoholic beverages.

Keywords—enzymatic determination; lactate; sodium bicarbonate-sodium hydroxide buffer; alcoholic beverage

Lactic acid is added to food as an acidulant and the decrease in pH of food due to the acidulant prevents contamination by various bacteria. Lactic acid is also used to enhance the flavor of sake or synthetic sake. The amount of lactic acid produced by alcoholic fermentation and the content of lactic acid in alcoholic beverages have to be determined for quality control. The AOAC method¹⁾ and the Davidson method²⁾ have been reported for the determination of lactate in foods. However, the enzymatic determination of lactic acid should be simpler than these methods.^{1,2)} The relevant reaction is: $\text{lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$, where NAD and NADH are nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide, reduced form, respectively, and LDH is lactate dehydrogenase. The amount of lactate present is proportional to the increase in absorbance of NADH at the wavelength of 340 nm. The equilibrium constant of the LDH reaction is $K = 2.7 \times 10^{11}$ M at 30°C and therefore, it strongly favors the formation of lactate and NAD^+ , instead of the

dehydrogenation of lactate by NAD^+ .³⁾ In order to drive the equilibrium to the right, glycine-hydrazine^{4,5)} or glutamate and glutamic-pyruvic transaminase (GPT)⁶⁻⁹⁾ have been added to the LDH reaction mixture. LDH, type II (rabbit muscle) also acts on other organic acids of similar structure, such as glycolic acid, oxalic acid, L-glutamic acid, D-isocitric acid, *etc.*, in glycine-hydrazine buffer.⁴⁾ However, it takes a long time (30 min at 37°C⁵⁾ or 40 min at 25°C⁹⁾) to reach equilibrium.

The purpose of this note is to describe a rapid determination method for lactate in alcoholic beverages in the absence of glycine-hydrazine or glutamate and GPT. Suitable conditions for the determination of lactate were investigated, such as selection of buffer, concentration and pH of buffer, concentration of NAD, reaction temperature and time.

Experimental

Reagents—(1) Buffer Solution: Glycine, sodium bicarbonate, borax, and orthoboric acid buffers were adjusted with sodium hydroxide. Orthoboric acid, borax, and sodium bicarbonate buffers were adjusted

with sodium carbonate. Ammonium chloride buffer was adjusted with ammonia. These buffers were 1.02 M solutions of pH 10.0.

(2) L- and D-Lactate Standard Solutions: L- and D-lithium lactate solutions of 32 mM.

(3) NAD Solution: β -NAD was obtained from Oriental Yeast Co., Ltd. and was 99.6% pure. A stock 64 mM solution was prepared in deionized and distilled water.

(4) Enzyme Solution: L-LDH (suspension, grade I from pig heart, 358 IU/mg) was obtained from Oriental Yeast Co., Ltd. D-LDH (suspension, from *Lactobacillus leichmannii*, ca. 300 IU/mg) was obtained from Boehringer Mannheim Corp. Solutions of 640 IU/ml of L- and D-LDH were prepared in deionized and distilled water.

(5) Sample Solution: Sake, shochu, wine, and whisky were commercial products. Sake (1→10), shochu (1→10), wine (1→20), and whisky (1→5) were diluted with water.

Procedure—Into a clean dry cuvette, 2.0 ml of buffer solution, 0.1 ml of the NAD solution, and 1.0 ml of the sample solution were pipetted and mixed. Then, 0.1 ml of L-LDH or D-LDH was added to the cuvette and mixed at 25°C for 2 min or 15 min, respectively. The absorbance (E_1) of the blank (water 1.0 ml) and that (E_2) of the sample solution were measured at the wavelength of 340 nm. Unknown concentrations in samples were determined from the calibration curves of L- and D-lactate.

Results and Discussion

1. Selection of Buffer

In order to find a suitable buffer for the LDH reaction, the absorbances of NADH at 340 nm in eight kinds of buffers were measured under the following conditions: NAD, 2 mM; L-lactate, 0.5 mM; L-LDH, 20 U/ml; concentration and pH of buffer, 200 mM and 10.0 in a cuvette at 25°C. The results obtained are shown in Table I. The absorbances of NADH produced by LDH reaction were zero in the borax and orthoboric acid buffers. The results may indicate that these buffers inhibit the

TABLE I. Comparison of Absorbance at 340 nm in Various Buffers

Buffer	ΔE^a
$\text{Na}_2\text{B}_4\text{O}_7\text{-NaOH}$	0.000
$\text{Na}_2\text{B}_4\text{O}_7\text{-Na}_2\text{CO}_3$	0.000
$\text{H}_3\text{BO}_3\text{-NaOH}$	0.000
$\text{H}_3\text{BO}_3\text{-Na}_2\text{CO}_3$	0.000
Glycine-NaOH	0.647
$\text{NH}_4\text{Cl-NH}_3$	0.733
$\text{NaHCO}_3\text{-NaOH}$	0.775
$\text{NaHCO}_3\text{-Na}_2\text{CO}_3$	0.655

a) $\Delta E = E_2 - E_1$; E_2 is the absorbance under the conditions, of 0.50 mM L-lactate, 200 mM buffer, and 2 mM NAD in a cuvette at pH, 10.0; E_1 is the absorbance of the blank.

enzyme reaction. Though the absorbances in glycine, ammonium chloride, and sodium bicarbonate buffers were approximately similar, the absorbance in the sodium bicarbonate-sodium hydroxide buffer was the highest, and thus the latter was chosen. It was stable for one month in a refrigerator.

2. Suitable Conditions for LDH Reaction

The absorbances of NADH obtained with 0.50 mM L- and D-lactate at buffer concentrations of 100, 200, 300, and 400 mM were measured. The absorbances in the range 300 to 400 mM were approximately constant. The

Fig. 1-A. Effect of Buffer pH on NADH Formation

●—●, with L-LDH; ▲—▲, with D-LDH.

Fig. 1-B. Effect of NAD Concentration on NADH Formation

●—●, with L-LDH; ▲—▲, with D-LDH.

Fig. 1-C. Effect of Reaction Time on NADH Formation

●—●, with L-LDH; ▲—▲, with D-LDH.

Fig. 1-D. Effect of Temperature on NADH Formation

●—●, with L-LDH; ▲—▲, with D-LDH.

optimum concentration was 400 mM. The effects of buffer pH, NAD concentration, reaction time, and temperature on NADH formation are shown in Fig. 1-A, 1-B, 1-C, and 1-D, respectively. The absorbance at pH 10.5 was greater than that at pH 10.0. However, the reaction was slow in the buffer of pH 10.5. The optimum pH was concluded to be 10.0.

Although the absorbance with 2.0 mM NAD was somewhat inferior to that with 4.0 mM, the NAD concentration was set at 2.0 mM in order to minimize expense. The amount of NAD used per one measurement was *ca.* one-third of that in the GPT method.⁹⁾

The times necessary to complete the L-LDH reaction and the D-LDH reaction were 2 min and 15 min, respectively. These reaction times are very short compared with those in other methods.^{5,9)}

The optimum temperature of the L- and D-LDH reactions was fixed at 25°C because of the thermostability of D-LDH.

3. Effect of Other Organic Acids on the LDH Reaction

Specificity for L-LDH was examined by employing similar organic acids (1.0 mM in a cuvette) such as glycolic acid, citric acid, and tartaric acid. The absorbances with these acids were 0.0017 in comparison with that (1.000) given by the same concentration of lactic acid. The results indicate that L-LDH is specific for lactic acid under the conditions used.

4. Recovery of Lactate

The amounts of L- and D-lactate contained in alcoholic beverages and the recoveries of added L- and D-lactate were examined by the procedure described in this note and are shown in Table II. The amounts of added lactate were 125, 250, and 500 mg/l. All the recoveries of L- and D-lactate from sake, shochu, wine, and whisky were in the range of 97.0 to

TABLE II. Recovery of Lactate from Alcoholic Beverages

Sample	Amount added (mg/l)	Found (mg/l)		Recovery (%) ^{a)}	
		L	D	L	D
Sake	0.0	204.7	262.3		
	125.0	331.5	395.8	101.4	106.8
	250.0	461.2	530.4	102.6	107.2
	500.0	700.5	778.3	99.2	103.2
Shochu	0.0	0.0	0.0		
	125.0	121.4	129.7	97.1	103.8
	250.0	252.2	251.3	100.9	100.5
	500.0	504.4	521.0	100.9	104.2
Wine	0.0	320.0	175.8		
	125.0	443.9	297.1	99.1	97.0
	250.0	565.0	432.4	98.0	102.6
	500.0	830.2	674.8	102.0	99.8
Whisky	0.0	7.2	0.0		
	125.0	138.9	129.6	105.4	103.8
	250.0	266.6	252.8	103.8	101.1
	500.0	518.9	509.0	102.3	101.8

a) Mean value of three determinations; coefficient of variation, 0.92.

107.2%. The present method, in which the LDH reaction is rapid compared with other methods,^{5,9)} was found to be practical for the determination of lactate in alcoholic beverages.

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