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The Determination of Kojic Acid Using the Stopped-flow Method[†]

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A kinetic method of the determination of kojic acid with 2,6-dichlorophenolindophenol(DCIP) is described. The oxidized form of DCIP, reacting with kojic acid, changes to the colorless reduced form of DCIP. By spectrophotometrically measuring the rate of discoloration, the apparent first-order rate constant, k_{app}, and the second-order rate constant, k, were obtained. The determination of kojic acid in the fermentation medium is possible by using k the constant, even in the presence of various organic substances.

Recently, kojic acid has attracted considerable attention in the food industry because it can be used as a starting material for synthesizing maltol and ethylmaltol, which are flavor enhancers of foods. It is often necessary to determine exactly the kojic acid which is produced in a fermentation medium. The colorimetric analysis of kojic acid with ammonium iron(III) sulfate has been widely used as a standard method[1–6] for the determination of kojic acid in a fermentation medium because of its simplicity and convenience, however, it involves some drawbacks in that it is susceptible to interference by the turbidity or color of the samples. Recently, the reaction of reductones with 2,6-dichlorophenolindophenol(DCIP) has been studied by Hiromi *et al.*,[7] Tonomura *et al.*,[8] and our laboratory[9–13] using the stopped-flow method, and its application to the determination of L-ascorbic acid and triose reductone has been suggested, since kojic acid is a pseudo-reductone whose redox behavior is quite similar to that of L-ascorbic acid.

Experimental

Materials. The DCIP is obtained from the Merck Chemical Co. Kojic acid was produced by the fermentation method using *Aspergillus oryzae* M-128.[14] It was purified by sublimation *in vacuo* at 130 °C. The other reagents used were of an analytical grade purchased from Wako Pure Chemical Industries, Ltd.

Measurement of the Rate Constant. The apparatuses used in this study were a Hitachi spectrophotometer Model 101 with a mixing injector and a Towa Model EPR-10A recorder. The reduction of DCIP was followed by measuring the absorbance at 525 nm,[13] the light path of the cell being 10 mm. The reactions were performed at 25 °C in a 0.2 mol dm^{-3} acetate buffer or a 0.2 mol dm^{-3} sodium phosphate buffer containing both 0.2 mol dm^{-3} sodium chloride and 5% 1-butanol, the DCIP was dissolved in distilled water containing 0.2 mol dm^{-3} sodium chloride and 5% 1-butanol. The concentration of the DCIP was kept at less than 5% of the concentration of kojic acid. The DCIP solution was prepared a fresh daily. All the kinetic results presented are the average of three measurements.

Results and Discussion

If we consider that the reaction of kojic acid with DCIP proceeds in a way similar to that of L-ascorbic acid, the reaction can be expected to obey the following second-order kinetics:

$$-d[\mathrm{DCIP}]/dt = k[\text{kojic acid}][\mathrm{DCIP}] = k_{\mathrm{app}}[\mathrm{DCIP}]$$

where [kojic acid] and [DCIP] represent the concentrations of kojic acid and DCIP respectively. When [kojic acid] is greatly in excess of [DCIP], k_{app} is the apparent first-order rate constant and k_{app} equals k times the initial concentration of kojic acid.

Reaction Curve of the Reaction of Kojic Acid with DCIP. Figure 1 shows the reaction curve of the reaction of kojic acid with DCIP. The reduction of DCIP was followed by the disappearance of the color at 525 nm for DCIP. In the initial stage of the reaction, a dead time due to the apparatus was observed. The apparent first-order rate constant(k_{app}) can be calculated from the absorbance changes by means of the following equation:[15]

$$\ln\,(A_0-A_\infty/A_X-A_\infty) = k_{\mathrm{app}}t$$

where A_0, A_X, and A_∞ represent the absorbances at $t=0$, X, and ∞ (the termination of the reaction) respectively. The first-order plot is linear, as is shown in Fig. 2. The apparent first-order rate constant, k_{app}, was obtained from the slope of the plot.

k-pH Profile. Figure 3 shows the pH dependence of the second-order rate constant, k. The second-order rate constants were obtained by dividing the k_{app} by the concentration of kojic acid. A maximum was observed in the acidic region (pH 6.0—6.5) for fast reactions. A bluff was observed at pH 7.0 for slow reactions. The k at pH 6.3 was about 5 times as large as that at pH 8.0. The half-time of the reaction, $t_{1/2}$, was 47.9 s for the kojic-acid concentration of 12.5 mmol dm^{-3} at pH 6.3. The plot of log k

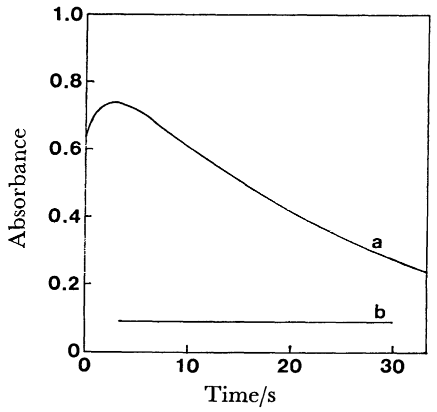

Fig. 1. Reaction curve of the reaction of kojic acid with DCIP at 25 °C.
pH 6.0, 10 mm cell, 525 nm, [kojic acid] = 100 mmol dm^{-3}, [DCIP] = 200 μmol dm^{-3}. a: Reaction signals, b: signals of the termination of reaction.

[†] This work was presented at the ACS/CSJ Chemical Congress, Honolulu, April 1979.

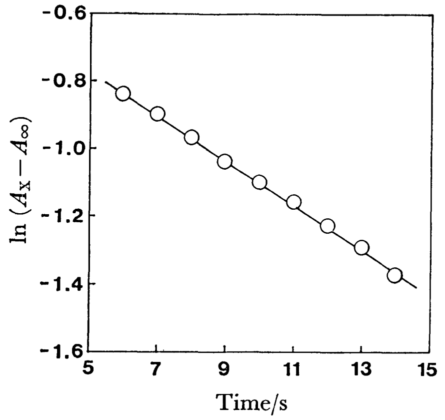

Fig. 2. First-order plot of the reaction of kojic acid with DCIP at 25 °C.
The experimental conditions were the same as in Fig. 1. A_X and A_∞ indicate the values of absorbances at time $=X$ and ∞ (the termination of reaction). It was obtained from the slope of the plot that k_{app} was 6.81×10^{-2} s^{-1}.

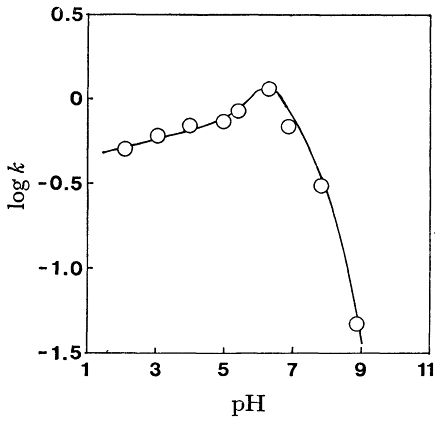

Fig. 3. The pH dependence of the second-order rate constants of the reaction of kojic acid with DCIP at 25 °C.
[Kojic acid] = 12.5 mmol dm^{-3}, [DCIP] = 200 μmol dm^{-3}, the ionic strength of the buffer solution was 0.10.

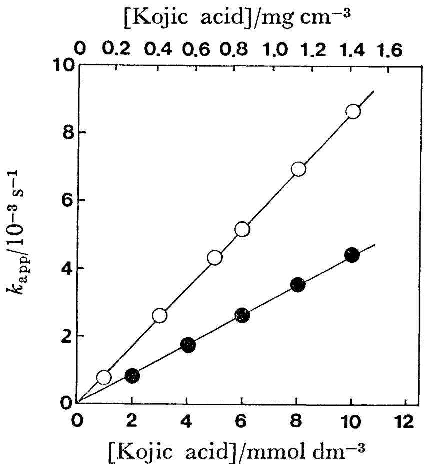

Fig. 4. Proportionally between k_{app} and kojic acid concentration at 25 °C.
[DCIP] = 200 μmol dm^{-3}, ●: pH 4.0, ○: pH 6.0.

TABLE 1. EFFECTS OF VARIOUS ADDED SUBSTANCES ON THE k OF THE REACTION OF KOJIC ACID WITH DCIP AT 25 °C

Added substance	$\frac{k}{\text{mol dm}^{-3}\text{ s}^{-1}}$	Relative rate constant
None	1.18	1.00
Glycine	1.13	0.96
L-Phenylalanine	1.18	1.00
L-Tryptophan	1.20	1.02
Malic acid	1.19	1.01
Succinic acid	1.19	1.01
Fructose	1.21	1.03
Glucose	1.13	0.96
Sucrose	1.19	1.01
$FeCl_3 \cdot 6H_2O$	38.6	32.7
$MgCl_2 \cdot 6H_2O$	1.21	1.03
$ZnCl_2$	1.18	1.00

pH 6.3, [kojic acid] = 12.5 mmol dm^{-3}, [DCIP] = 200 μmol dm^{-3}, [Added substance] = 12.5 mmol dm^{-3}.

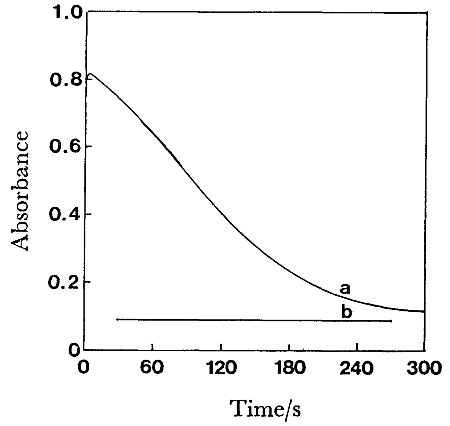

Fig. 5. Reaction curve of the reaction of DCIP with kojic acid in the fermentation medium at 25 °C. pH 6.0, [DCIP] = 200 μmol dm^{-3}. a: Reaction signals, b: signals of the termination of reaction. The growth medium were glucose (10.0 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), KH_2PO_4 (0.1 g), and bonito extract (0.25 g). The culture was shaken on a rotary shaker for 72 h at 25 °C.

against the pH is different from that obtained for the reaction of L-ascorbic acid with DCIP. These results suggest that the reaction mechanism of kojic acid with DCIP is considerably different from that of L-ascorbic acid over a wide pH range.

Calibration Curve of Kojic-acid Determination. Figure 4 shows the proportionality between k_{app} and the kojic-acid concentration at pH 4.0 and 6.0. It gives a straight line, suggesting the possibility of applying the reaction to the determination of kojic acid. The determination of kojic acid using the stopped-flow method was possible, even in the pH 2—7 region and in concentrations from 1 to 100 mmol dm^{-3}.

Effects of Various Substances. Table 1 shows the effects of various substances often encountered in kojic-acid determination; amino acids, organic acids, sugars, and metal ions were tested. The results show that

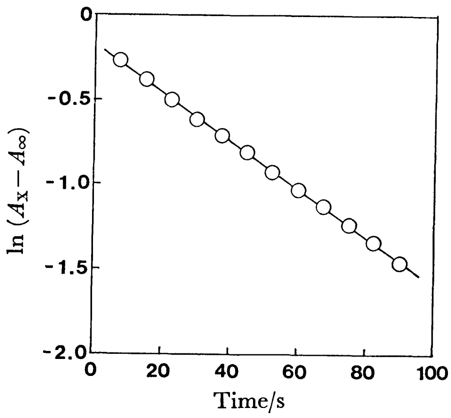

Fig. 6. First-order plot of the reaction of DCIP with kojic acid in the fermentation medium at 25 °C. The experimental conditions were the same as in Fig. 5. It was obtained from the slope of the plot that k_{app} was $1.41 \times 10^{-2}\ s^{-1}$.

most additives, except for iron(III) chloride, do not interfere with the determination of kojic acid. The iron(III) chloride, however, reacted with kojic acid.[1)]

Analysis of Kojic Acid in the Fermentation Medium. The growth medium of the mold strain of *Aspergillus* was prepared with glucose (10.0 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), KH_2PO_4 (0.1 g), and bonito extract (0.25 g). The culture was shaken on a rotary shaker for 72 h at 25 °C. Figure 5 shows the results of the analysis of the kojic-acid fermentation medium containing various substances. The apparent first-order rate constant (k_{app}) was obtained from the slope of the apparent first-order plot of Fig. 6. In this method, the kojic-acid concentration in broth was determined to be 18.0 mg cm^{-3}. By the colorimetric method, the amount of kojic acid was determined to be 18.3 mg cm^{-3}. The analytical data obtained by the stopped-flow method is in fair agreement with the colorimetric data. The determination of kojic acid using the stopped-flow method may be recommended for use with colored and turbid samples such as the fermentation medium of molasses.

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