Studies on Kojic Acid Metabolism by Microorganisms

Part X. Kojic Acid Oxidase

By Jun IMOSE, Seiichi NONOMURA and Chuji TATSUMI

Laboratory of Agricultural Technology, Department of Agriculture,
University of Osaka Prefecture, Sakai, Osaka, Japan

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An enzymatic oxidation of kojic acid to comenic aldehyde was found in the decomposition process of kojic acid by *Arthrobacter ureafaciens* strain (K-1), a kojic acid decomposing bacteria.

This enzyme was (probable a new type of non-heme iron protein) is assumed to catalyze the dehydrogenation of kojic acid, while the ferric ion contained in the enzyme is considered to serve as an acceptor of hydrogen released from kojic acid. The resulted ferrous ions are oxidized either by molecular oxygen under aerobic conditions or by NAD under anaerobic conditions, accompanying hydrogen peroxide in the former and reduced NAD in the latter.

The enzyme was partially purified by using ammonium sulfate precipitation, gel filtration on Sephadex G-200 column and column chromatography with DEAE-Sephadex A-50. The activity increased to 85 fold, compared with crude extracts and the recovery of the activity was 33.9%. The optimum pH of the reaction was 7.75. The enzyme was inactivated by PCMB, and unstable upon heat treatment. A loss of about 50% of the activity was caused by heating at 35°C for 5 min, but some reducing agents protected the enzyme from PCMB inhibition and the heat inactivation. Not only kojic acid, but also benzyl kojic acid or 5-methoxy kojic acid may be substrates. Km value for kojic acid was $1.43 \times 10^{-5}$ m. The molecular weight of the enzyme was estimated to be about 55,000 and the enzyme contained about two atoms of iron in one molecule. The reaction mechanism for kojic acid oxidase is discussed.

In the course of our studies on kojic acid degradation by microorganisms, *Arthrobacter ureafaciens* strain (K-1) was isolated from soil as a microorganism to utilize kojic acid as a sole carbon source and some of the intermediates involved were identified from the reaction mixture.1) As the results of degradation of kojic acid by resting cells, we proposed a degradation pathway as follows; Kojic acid $\rightarrow$ Comenic aldehyde $\rightarrow$ Comenic acid $\rightarrow$ D-Galacturonic acid $\rightarrow$ D-Tagaturonic acid $\rightarrow$ TCA cycle. In this pathway of D-galacturonic ketol isomerase, we already reported a partial purification and some properties of the enzyme.2,3)

This paper deals with partial purification and some properties of kojic acid oxidase, which forms comenic aldehyde by oxidation in hydroxymethyl group of kojic acid when strain (K-1) is grown with kojic acid as a sole carbon source.

MATERIALS AND METHODS

Microorganism. *Arthrobacter ureafaciens* strain (K-1)

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was maintained on the 0.2% kojic-bouillon agar.

Cultural conditions. In static culture, strain (K-1) was grown in 500 ml volume flask containing 200 ml of 0.2% kojic-bouillon medium consisting of meat extracts, 0.5%; peptone, 1.0%; NaCl, 0.5%; all adjusted to pH 7.2 with KOH, and 0.2% kojic-synthetic medium consisting of KH₂PO₄, 0.2%; NH₄NO₃, 0.1%; MgSO₄·7H₂O, 0.05%; yeast extracts, 0.025%; all adjusted to pH 7.2 with KOH. After sterilization, 1 ml inoculum of overnight culture was added and then the static culture was incubated at 30°C for 70 hr and the shaking culture was incubated at 30°C for 24 hr.

Enzyme assay. The enzyme activity was followed by measuring the rate of increase in the absorbance at 335 nm as described below, in a 1.0 cm light path at room temperature with Shimazu model QV-50 spectrophotometer and Nippon Denshi Co. Ltd., Unicorder U-100. Unless otherwise specified, the reaction mixture contained following constituents in a final volume of 3.0 ml: 100 mM of potassium phosphate buffer, pH 7.8; 3 mM of kojic acid; enzyme solution. Under these conditions, the rate of reaction was proportional to the enzyme concentration during initial 3 min. Enzyme activity was measured as the rate of change in absorbancy at 335 nm per 3 min per mg protein. The basis of this determination method will be discussed later.

Protein concentration. Protein concentration was estimated spectrophotometrically by the method of Warburg and Christian.⁴

Iron determination. Iron was determined by the o-phenanthroline method of Harvey et al.⁵ The sample, enzyme solution, to be analysed was heated for 5 min at 80°C in 1% HCl to liberate labile sulfur in polyethylene centrifuge tubes. After prompt cooling, the sample in the tube was deproteinized by centrifugation, the supernatant was transferred to a 10 ml volumetric flask, and the precipitate was treated by the same procedure again, then the supernatant thus obtained was transferred to another 10 ml volumetric flask. To each aliquot, were added 2.0 ml of 0.2 M potassium biphthalate buffer, pH 3.95, 1.0 ml of 10% of hydroxylamine and 4.0 ml of 0.3% o-phenanthroline aqueous solution, then the flask was filled up to 10 ml with distilled water. The absorbancy of iron-o-phenanthroline complex was measured at 512 nm. A standard curve was obtained using ferrous ammonium sulfate hexahydrate. Total iron was determined as ferrous ion under the condition in which ferric ion was reduced. Sum of the two assays is presented as relative value of total iron.

Molecular weight. Molecular weight of kojic acid oxidase was estimated by thin-layer gel filtration with Sephadex G-200.⁶ The flow of 0.05 M potassium phosphate buffer containing 0.5 M NaCl through the layer was maintained for a day before application of samples. The enzyme protein was applied on the plate with standard proteins, and developed for 8 hr. When development was considered complete, the plate was removed from development chamber, and then Toyo Roshi No. 50 filter paper was applied immediately to gel surface. The covered plate was transferred to an oven for drying, and stained with amidoschwartz in 7% acetic acid, followed by extensive washing with water and 7% acetic acid to remove excess dye. The distance between the starting point and center of each spot was measured.

Estimation of oxygen uptake. Oxygen uptake was estimated by Warburg’s manometer.

Reagents. Kojic acid used for culturing of micro-organism was purified by recrystallization from ethanol. For the enzyme reaction, kojic acid was further purified by sublimation. 5-Methoxy kojic acid was recrystallized from ethanol after being prepared by ordinary method. 5-Methoxy comenic aldehyde was prepared by Becker’s method.⁷ o-Phenanthroline (Wako pure chemical industries, Ltd.), chymotrypsinogen (Tokyo Kasei Kogyo Co., Ltd.), horseradish peroxidase (Boehringer and Soehne DmbH, Mannheim) bovine serum albumin (Fraction V) (Armour Laboratories and Co., Ltd.) and human γ-globulin (Fraction II) (Tokyo Kasei Kogyo Co., Ltd.) were obtained from commercial sources.

RESULTS

1) Purification of kojic acid oxidase

The results of the preparation of kojic acid

⁴ O. Warburg and Christian, Biochem. Z., 310, 384 (1941).
⁵ A. E. Harvey, J. A. Smart, Jr. and E. S. Amis, Analyt. Chem., 27, 26 (1955).
TABLE I. PURIFICATION OF KOJIC ACID OXIDASE

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units$^{(1)}$)</th>
<th>Specific activity (units$^{(1)}$/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract</td>
<td>40</td>
<td>908</td>
<td>11,800</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation (20~40% sat.)</td>
<td>5</td>
<td>80.8</td>
<td>11,200</td>
<td>138</td>
<td>95</td>
</tr>
<tr>
<td>Sephadex G-200 gel filtration</td>
<td>15</td>
<td>21.6</td>
<td>6,500</td>
<td>300</td>
<td>55</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 fractionation</td>
<td>35</td>
<td>3.38</td>
<td>4,000</td>
<td>1020</td>
<td>33.9</td>
</tr>
</tbody>
</table>

$^{(1)}$ 0.001 O.D.355/3 min/mg protein = 1 unit.

oxidase are summarized in Table I. All steps were performed at 0~5°C.

**Step 1. Preparation of cell extracts.** Cells in culture medium were harvested by centrifugation at 5000 × g for 5 min, washed twice with sterilized water, and suspended in 1/15 M potassium phosphate buffer, pH 7.8. The cells were disrupted by sonication for 15 min, followed by centrifugation at 12,000 × g for 10 min, and the supernatant was used as cell extracts.

**Step 2. Ammonium sulfate fractionation.** Solid ammonium sulfate was added to the solution obtained in Step 1 so as to be 20% saturation under stirring, stirring was continued for 20 min, and precipitate was removed by centrifugation at 12,000 × g for 10 min. Solid ammonium sulfate was further added to 40% saturation. After centrifugation, the precipitate having an enzyme activity was dissolved in 1/20 M potassium phosphate buffer, pH 7.8, and the enzyme solution was used as ammonium sulfate fraction.

**Step 3. Sephadex G-200 gel filtration.** A column (1.8 × 45.0 cm) was packed with Sephadex G-200 in 1/20 M potassium phosphate buffer, pH 7.8, and repeatedly washed with the same buffer. The enzyme solution obtained in Step 2 was applied to the column and eluted with the above buffer at a flow rate of 0.25 ml/min. The protein concentration and enzyme activity determined for this system are shown in Fig. 1. The active effluent fractions were combined and used as Sephadex G-200 fraction.

**Step 4. DEAE-Sephadex A-50 fractionation.** The enzyme obtained in Step 3 was applied to a DEAE-Sephadex A-50 column (1.8 × 33.0 cm) previously equilibrated with 1/20 M potassium phosphate buffer, pH 7.8. A linear gradient was applied with 200 ml of the buffer, pH 7.8, in the mixing flask and 500 ml of the same buffer, 0.5 M with respect to KCl, in a reservoir. Each 5 ml fraction was collected at a rate of approximately 0.4 ml per min. The elution pattern is shown in Fig. 2. The most active fractions were pooled and used as DEAE-Sephadex A-50 fraction.

**II) Induction of kojic acid oxidase**
Specific activities of kojic acid oxidase in...
FIG. 2. Chromatography on a DEAE-Sephadex A-50 Column.

--- Protein.
--- Kojic acid oxidase activity.
--- KCl.

Sephadex G-200 fraction prepared from strain (K-1) which was grown on various culture media and under various culture conditions are shown in Table II. The highest specific activity is seen in kojic-synthetic medium with static cultural condition. This was therefore chosen for the preparation of kojic acid oxidase.

III) Characteristics of kojic acid oxidase as non-heme iron protein

1. Properties of kojic acid oxidase as non heme iron protein

Enzyme solution used in the experiments described below was Sephadex G-200 fraction.

a) Absorption spectra of enzyme protein. The enzyme protein showed a single absorption peak at 278 m\(\mu\) and a small peak at near 410 m\(\mu\). When concentrated by colodion bag, the latter showed a characteristic absorption band at 408 m\(\mu\) (Fig. 3). The concentrated enzyme solution was deproteinized by addition

FIG. 3. Absorption Spectra of Kojic Acid Oxidase.

The enzyme solution (Sephadex G-200 fraction) was incubated in a standard reaction mixture at 20°C for 3 min.

--- Native enzyme.
--- Reduced enzyme with dithionite.
--- Oxidized enzyme with aeration for 10 min after reduction.

<table>
<thead>
<tr>
<th>Media</th>
<th>Culture condition</th>
<th>Cell growth (g wet wt./liter)</th>
<th>Activity (O.D.\textsubscript{545}/3 min/mg protein)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic medium</td>
<td>Static culture</td>
<td>1</td>
<td>0.414</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Shaking culture</td>
<td>5</td>
<td>0.0234</td>
<td>5.65</td>
</tr>
<tr>
<td>Bouillon medium</td>
<td>Static culture</td>
<td>2</td>
<td>0.0939</td>
<td>21.95</td>
</tr>
<tr>
<td></td>
<td>Shaking culture</td>
<td>10</td>
<td>0.0513</td>
<td>12.39</td>
</tr>
</tbody>
</table>

Static culture incubated at 30°C for 70 hr.
Shaking culture incubated at 30°C for 24 hr.
The enzyme activity was determined in a standard reaction mixture under the standard assay condition. Sephadex G-200 fraction was used as the enzyme solution.
of trichloroacetic acid or HCl in final concentration of 5%, and in this solution ferrous iron could be detected qualitatively.

b) Disappearance of 408 m\(\mu\) absorption band by dithionite and recovery by reoxidation. It has been known\cite{8,9,10} that a characteristic absorption band of non-heme iron protein disappears when it is reduced by dithionite. The absorption peak at 408 m\(\mu\) of the present enzyme also disappears after addition of a small amount of dithionite, but appeared again upon oxidation by aeration for 10 min as shown in Fig. 3. It is of interest to compare an absorption spectrum of reduced enzyme with that of native (oxidized) one. This change may be due to the state of iron contained in the enzyme protein. Figure 4 shows difference spectrum between the reduced and the native (oxidized) enzyme.

c) Reduction of the enzyme by hydrogen released by the enzyme reaction. At first, an absorption spectrum of the native enzyme was determined enzimically reduced enzyme was prepared as follows. After incubation for 2 hr under anaerobic conditions with kojic acid, the enzyme was rapidly treated with ammonium sulfate solution of 40% saturation, the precipitate was centrifuged and dissolved in 5 ml of 1/20 M potassium phosphate buffer, pH 7.8. As shown in Fig. 5, an absorption peak at 408 m\(\mu\) was observed to disappear both of the enzymatically reduced enzyme and of the reduced enzyme by dithionite treatment. The reduced enzyme was oxidized by aeration, and the spectrum of the reoxidized enzyme thus obtained coincided with the corresponding spectrum with the dithionite treatment (Fig. 5). It may be mentioned here that iron contained in the enzyme is in ferric state and that reduction to the ferrous state by hydrogen released from kojic acid can occur.

d) Role of NAD and thionine as hydrogen acceptor of reduced kojic acid oxidase. It is
presented in Fig. 6 and Table III that NAD and thionine may serve as electron acceptor in anaerobic conditions. When iron contained in enzyme protein was almost reduced, an absorbancy at 335 mµ could be observed to increase again in addition of NAD in the incubation mixture.

**e) Determination of iron.** Amount of iron in enzyme protein was measured quantitatively (the most active fraction on Sephadex G-200 column chromatography was assayed) by o-phenanthroline method. Iron content of 0.5 µg was found per mg protein.

**f) Determination of molecular weight and number of iron atoms.** Molecular weight of kojic acid oxidase was determined by thin layer Sephadex G-200 gel chromatography. As standard protein, four proteins of known molecular weight were used: Chymotrypsinogen (mol. wt. 25,000), horseradish peroxidase (mol. wt. 40,000), bovine serum albumin (mol. wt. 67,000) and γ-globulin (mol. wt. 160,000). These four proteins and kojic acid oxidase (Sephadex G-200 fraction concentrated by colodion bag) were developed at the same time and molecular weight was estimated to be about 55,000 from migration.

![Graph showing reduction of NAD](image)

**FIG. 6. Reduction of NAD.**

Reaction mixture contained 100 µmoles of potassium phosphate buffer (pH 7.8), 3 µmoles of kojic acid and enzyme solution (Sephadex G-200 fraction) 0.5 ml in total volume of 3.0 ml. The enzyme activity at start of incubation was 0.137 (O.D.335/3 min/0.398 mg protein). After 60 min, the remaining activity was 0.0020, and after 63 min, 0.5 µmoles of NAD was added.

**TABLE III. REDUCTION OF THIONINE**

<table>
<thead>
<tr>
<th>Phosphate buffer (pH 7.8) µmole</th>
<th>Kojic acid µmole</th>
<th>Thionine µmole</th>
<th>Enzyme ml</th>
<th>Reduction time min</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>5</td>
<td>2</td>
<td>1.0</td>
<td>125</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>2</td>
<td>—</td>
<td>&gt;360</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>2</td>
<td>1.0</td>
<td>&gt;360</td>
</tr>
</tbody>
</table>

Reaction mixture showed above (total volume of 3 ml) was incubated at 30°C in dark place in Thunberg tube.

a) Activity of enzyme (Sephadex G-200 fraction) was 0.1079 (O.D.335)/0.465 mg protein/3 min, and the enzyme solution contained 0.990 mg protein.

**FIG. 7. Thin-layer Chromatogram of Kojic Acid Oxidase on Sephadex G-200.**

distance (Fig. 7). The enzyme was found to contain 0.5 µg of iron per mg protein, from which molecular weight was calculated 110,000. As it was 55,000 on the basis of TLC, it was assumed that the enzyme contains about two atoms of iron in one molecule of the protein.

g) A new absorption peak at 335 mµ appeared during reaction of dehydrogenation of kojic acid oxidase. (Experimental basis for determination of kojic acid oxidase activity.) From previous report and the result 2(b) and (c), it may be suggested that iron contained in the enzyme protein was reduced to ferrous state by the hydrogen released by dehydrogenation of kojic acid. An absorption peak at 333 mµ due to the formation of a ferrous iron-comenic aldehyde complex (Fig. 8) will appear. This complex is especially formed only in the presence of iron as ferrous state in enzyme protein, and although the complex of ferrous sulfite-kojic acid usually shows yellow color, it does not exhibit an absorption peak in ultraviolet region. From these phenomena it is possible to estimate kojic acid oxidase activity. Relation between enzyme concentration and enzyme activity is shown in Fig. 9 in case of Sephadex G-200 fraction, and in Fig. 10 in case of DEAE-Sephadex A-50 fraction. In both cases, activity of the enzyme was proportional to the absorption at 335 mµ within a certain concentration range. This method can be applied only to 5-hydroxy 7-pyrone compounds, but not to 5-methoxy kojic acid which is oxidized by kojic acid oxidase.

IV) Properties of kojic acid oxidase
DEAE-Sephadex A-50 fraction was used as an enzyme solution in the following experiment.

a) Optimum pH. The assay for kojic acid
FIG. 10. Kojic Acid Oxidase Activity as a Function of Enzyme Concentration.

The reaction mixture contained 100 μmoles of potassium phosphate buffer (pH 7.8), 3 μmoles of kojic acid and various volumes of enzyme solution (DEAE-Sephadex A-50 fraction) in total volume of 3 ml, and was incubated at 20°C for 3 min. Straight line shows kojic acid oxidase activity as a function of enzyme activity calculated by method of least minimum square.

oxidase activity was performed in a potassium phosphate buffer system at pH value between 6.5 and 8.0, and in borate-potassium phosphate buffer between pH 7.75 and 9.0. As shown in Fig. 11, the optimum pH for the initial reaction is 7.67 in the former buffer system, and 7.75 in the latter.

b) Stability for heating. Enzyme was rapidly inactivated by heating, a 55 per cent loss of the activity was caused by heating at 35°C for 5 min, and the activity was almost perfectly lost at 50°C in 5 min.

c) Protective effect of various reagents against heat inactivation of the enzyme. As a loss of activity occurred by heat treatment at 35°C for 5 min, protective effects of various reducing agents shown in Table IV were inves-

FIG. 11. Effect of pH on the Activity of Kojic Acid Oxidase.

The enzyme solutions (DEAE-Sephadex A-50 fraction) were incubated at various pH values in a standard reaction mixture. Each pH value represents final pH.

- - Potassium phosphate buffer.
- - Borax-KH₂PO₄ buffer.

FIG. 12. Effect of Temperature on the Activity of Kojic Acid Oxidase.

The enzyme solutions (DEAE-Sephadex A-50 fraction) were treated at various temperatures for 5 min, and after cooling, they were incubated in a standard reaction mixture.
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TABLE IV. PROTECTIVE EFFECT OF REDUCING AGENTS AGAINST ENZYME INACTIVATION BY HEAT TREATMENT

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>Conc. (μmole)</th>
<th>Relative activity remained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Heat</td>
<td>Dithiothreitol</td>
<td>3</td>
<td>80.0</td>
</tr>
<tr>
<td>treatment</td>
<td>”</td>
<td>6</td>
<td>79.4</td>
</tr>
<tr>
<td></td>
<td>Mercaptoethanol</td>
<td>12</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>L-Cysteine</td>
<td>60</td>
<td>101.6</td>
</tr>
<tr>
<td></td>
<td>Glutathione</td>
<td>60</td>
<td>45.5</td>
</tr>
</tbody>
</table>

Before heat treatment, various reducing agents were added to the enzyme solution, and they were heated at 35°C for 5 min. After cooling, the remaining activity was determined. DEAE-Sephadex A-50 fraction was used as the enzyme solution.

tigated. Dithiothreitol and mercaptoethanol exerted protective action against heat inactivation of the enzyme, but cysteine and glutathione were much less effective.

d) Protective effect of various reagents against inactivation by low temperature storage. A 35.5 per cent loss of activity occurred upon storage at 5°C for 48 hr, but this inactivation was significantly prevented by the addition of mercaptoethanol and dithiothreitol (Table V). These substances were thus found to be favorable for the storage of the enzyme.

TABLE V. EFFECT OF REDUCING AGENTS ON THE STABILITY OF KOJIC ACID OXIDASE

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>83.2</td>
</tr>
<tr>
<td>Enzyme + Mercaptoethanol (60 μmole)</td>
<td>93.3</td>
</tr>
<tr>
<td>Enzyme + Dithiothreitol (3 μmole)</td>
<td>87.2</td>
</tr>
</tbody>
</table>

The enzyme solutions (DEAE-Sephadex A-50 fraction) containing each reducing agent were preserved at 5°C for each time, and then the enzyme solutions were incubated in a standard reaction mixture.

e) Effect of metal ions. None of the metal ions presented in Table VI stimulated the activity of the enzyme.

TABLE VI. EFFECT OF METAL ION

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Conc. (μmole)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>3</td>
<td>94.5</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>3</td>
<td>92.5</td>
</tr>
<tr>
<td>CoSO₄</td>
<td>3</td>
<td>84.7</td>
</tr>
</tbody>
</table>

Each metal ion was added to the standard reaction mixture, and it was incubated at room temperature for 3 min. DEAE-Sephadex A-50 fraction was used as the enzyme solution. The activity was determined under the standard assay condition.

f) Effect of metal binding agents. Although no requirement of metal ions was observed, α-α’ dipyrindyl, benzoyl acetone, citrate and oxalate were found markedly inhibitory (Table VII). In this experiment, Sephadex G-200 fraction was used as the enzyme solution.

TABLE VII. EFFECT OF METAL-BINDING AGENTS

<table>
<thead>
<tr>
<th>Metal-binding agents</th>
<th>Conc. (μmole)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>α-α’ Dipyrindyl</td>
<td>6</td>
<td>100.0</td>
</tr>
<tr>
<td>Benzoyl acetone</td>
<td>6</td>
<td>100.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>6</td>
<td>93.0</td>
</tr>
<tr>
<td>Oxalate</td>
<td>6</td>
<td>90.8</td>
</tr>
<tr>
<td>α-Phenanthonline</td>
<td>6</td>
<td>36.0</td>
</tr>
<tr>
<td>8-Hydroxy quinoline</td>
<td>6</td>
<td>23.4</td>
</tr>
<tr>
<td>EDTA</td>
<td>30</td>
<td>83.0</td>
</tr>
</tbody>
</table>

The enzyme reactions were carried out in a standard reaction mixture containing each metal-binding agent. DEAE-Sephadex A-50 fraction was used as the enzyme solution.

g) Effect of various inhibitors. The enzyme activity was considerably inhibited by PCMB (Table VIII). Other reagents tested had no significant effect, but inhibition by PCMB
suggested that sulfhydryl group may be involved in the reaction catalyzed by the enzyme.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Conc. (µmole)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.3</td>
<td>19.3</td>
</tr>
<tr>
<td>MIA</td>
<td>0.3</td>
<td>79.2</td>
</tr>
<tr>
<td>Acetamide</td>
<td>0.3</td>
<td>89.8</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.3</td>
<td>89.8</td>
</tr>
<tr>
<td>Arsenate</td>
<td>0.3</td>
<td>91.3</td>
</tr>
<tr>
<td>Arsenite</td>
<td>0.3</td>
<td>91.3</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0.3</td>
<td>91.3</td>
</tr>
</tbody>
</table>

The enzyme reactions were carried out in a standard reaction mixture containing each inhibitor. DEAE-Sephadex A-50 fraction was used as the enzyme solution.

h) Protective effect of L-cysteine against PCMB inhibition. PCMB inhibition was eliminated when L-cysteine had previously been added in the reaction mixture (Table IX), and these results suggest that sulfhydryl group was protected by L-cysteine from inactivation by PCMB.

i) Substrate specificity. Enzyme activity was determined toward a few kojic acid derivatives, sugar alcohol and aromatic alcohol on the basis of oxygen uptake by Warburg's manometer. These results are tabulated in Table X. The enzyme could react with benzyl kojic acid and 5-methoxy kojic acid. It was suggested that in view of similarity of their structures with kojic acid, the enzyme was able to oxidize hydroxymethyl group adjacent to γ-pyrone ring to a corresponding aldehyde group. It showed no significant activity towards benzyl alcohol, but reacted more or less with anise alcohol.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. (µmole)</th>
<th>µl O₂ uptake/60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kojic acid</td>
<td>10</td>
<td>106.8</td>
</tr>
<tr>
<td>5-Methoxy kojic acid</td>
<td>10</td>
<td>156.5</td>
</tr>
<tr>
<td>Benzyl kojic acid</td>
<td>10</td>
<td>94.4</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>10</td>
<td>14.6</td>
</tr>
<tr>
<td>Anise alcohol</td>
<td>10</td>
<td>48.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10</td>
<td>16.2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10</td>
<td>11.0</td>
</tr>
<tr>
<td>Arbutol</td>
<td>10</td>
<td>5.6</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>10</td>
<td>3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>1. (L-Cysteine + PCMB + Enzyme) + Kojic acid</td>
<td>89.5</td>
</tr>
<tr>
<td>2. (PCMB + Enzyme) + L-Cysteine + Kojic acid</td>
<td>19.3</td>
</tr>
</tbody>
</table>

Experiment 1 indicates that the enzyme solution was preincubated with L-cysteine and PCMB, and then the enzyme reaction was started by the addition of kojic acid.

Experiment 2 indicates that the enzyme solution was preincubated with PCMB, and then the enzyme reaction was started by addition of L-cysteine and kojic acid.

In each experiment, DEAE-Sephadex A-50 fraction was used as the enzyme solution, and the concentrations were 3 µmoles for L-cysteine, 0.3 µmoles for PCMB and 3 µmoles for kojic acid.

j) Michaelis constant. The apparent Michaelis constant of kojic acid oxidase for kojic acid was found to be $1.43 \times 10^{-5}$ M by method of Lineweaver and Burk (Fig. 13).

V) Identification of reaction products

a) Paper chromatography of reaction product. Reaction mixture contained 2.0 mmoles of
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**FIG. 13. Lineweaver-Burk Plot for Kojic Acid Oxidase.**

The enzyme solution (DEAE-Sephadex A-50 fraction) was incubated at pH 7.8 for 3 min in a standard reaction mixture with each concentration of substrate. Substrate concentration represents the final concentration in a standard reaction mixture.

![Lineweaver-Burk Plot](image)

Potassium phosphate buffer pH 7.8, 200 μmoles of kojic acid and 20 ml of enzyme solution (Sephadex G-200 fraction) in total volume of 60 ml. Buffer and kojic acid were first mixed, followed by bubbling N₂ for 3 min, and enzyme solution was added to the mixture and again N₂ was bubbled for 3 min. After the reaction vessel was stoppered, the reaction mixture was incubated for 4.5 hr at room temperature. At the end of the reaction, 1 ml of aqueous sulfuric acid (H₂O : H₂SO₄ = 1 : 3) was added, and reaction mixture was extracted with ether for 24 hr. The dried substance obtained with ether extraction was dissolved in a small volume of ethanol. Separation of the sample with paperchromatography using n-butanol-acetic acid-water (5 : 2 : 3, v/v) and detection of spots by spraying with ferric chloride were carried out, and Rf values of two compounds were 0.65 and 0.54 (Fig. 14). A Rf value of 0.54 is identical with that of kojic acid, and a Rf value of 0.65 seems highly probable to demonstrate spot of reaction product, comenic aldehyde.

**b) Formation of 5-methoxy comenic aldehyde semicarbazone.** Activity of kojic acid oxidase was not affected by the addition of 10⁻³ to 10⁻⁴ M semicarbazide in standard incubation mixture. When kojic acid was replaced by 5-methoxy kojic acid, oxidation of the latter by the enzyme yielded a product with a semicarbazone derivative. Reaction mixture was as follows; 100 μmoles of potassium phosphate buffer (pH 7.8), 10 μmoles of 5-methoxy kojic acid, 8 μmoles of semicarbazide and 1.0 ml of enzyme solution (Sephadex G-200 fraction, 1.957 mg protein) in a total volume of 3 ml. It was aerated for 2 hr at room temperature, and at the end of reaction it was deproteinized by addition of 0.1 ml of aqueous sulfuric acid (H₂O : H₂SO₄ = 1 : 3). As shown in Fig. 15, absorption spectrum of semicarbazone in reaction mixture was identified with that of corresponding derivative of 5-methoxy comenic aldehyde.
FIG. 15. Ultraviolet Absorption Spectra of Semi
carbazone of 5-Methoxy Comenic Aldehyde and
the Reaction Product.

Reaction mixture: 100 μmoles of potassium
phosphate buffer (pH 7.7), 10 μmoles of 5-methoxy
kojic acid, 8 μmoles of semicarbazide and 1.0 ml
of enzyme solution (Sephadex G-200 fraction) in
total volume of 3.0 ml.

a: 5-Methoxy comenic aldehyde.
b: Reaction product.

c) Formation of hydrogen peroxide. Formation
of hydrogen peroxide was assayed by
Farmer's method,11 and detected by the ap-
pearance of red-brown color when o-dianisidine
and peroxidase were present. Some of the
results are shown in Table XI. Although
control solution without kojic acid had an
absorbancy of 0.065 at 420 mμ, the test sol-
ution had that of 0.500. This result indicates
the formation of hydrogen peroxide as a re-
action product. Furthermore, oxygen uptake
was 90 μl under aerobic conditions and there
was no oxygen uptake under anaerobic con-
ditions (replacement by N2) when it was esti-
inated with manometric procedure.

<table>
<thead>
<tr>
<th>TABLE XI. FORMATION OF HYDROGEN PEROXIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction mixture: 2.0 mg of peroxidase and 0.5 ml of 1%</td>
</tr>
<tr>
<td>(w/v) o-dianisidine in 95% (w/v)</td>
</tr>
<tr>
<td>ethanol, in 29.5 ml of phosphate buffer,</td>
</tr>
<tr>
<td>pH 7.8</td>
</tr>
<tr>
<td>Kojic acid</td>
</tr>
<tr>
<td>3.0 μmoles</td>
</tr>
<tr>
<td>Enzyme solution (Sephadex G-200 fraction)</td>
</tr>
<tr>
<td>1.0 ml</td>
</tr>
<tr>
<td>Total volume</td>
</tr>
<tr>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

Reactions were carried out at room temperature for
15 min.

11) V. C. Farmer, M. E. K. Henderson and J. D.

DISCUSSION

It is generally recognized that the electron
acceptor for dehydrogenase is pyridine or
flavine nucleotide, but the experimental results
indicate that these coenzymes are not involved
in kojic acid oxidase.

An absorption peak at 408 mμ is character-
istic to this enzyme. In general, enzyme
proteins which have chromophoric group are
usually known as heme protein, iron flavo-
protein and iron protein. It is characteristic
of non-heme iron protein that an absorption
peak in visible region disappears upon redu-
ction with dithionite and is recovered on re-
odification. When this enzyme (Sephadex G-
200 fraction) was reduced with dithionite or
incubated with kojic acid, the absorption peak
at 408 mμ disappeared, and was restored by
aeration. Judging from the absorption peak
in visible region, this enzyme protein is nei-
ther heme protein nor iron flavoprotein, but
shows properties similar to those of non-heme
iron proteins. Although an absorption spec-
trum of this enzyme in visible region is
slightly different from those of some non-
heme iron proteins, it is probably due to the
difference of origin and that of amino acid
composition, etc.

As is postulated from the electric potential
of iron, iron is easy to be oxidized by oxygen,
and hence it may be in the most stable, ferric
state in vivo. In the present enzyme solution
concentrated by collodion bag, the presence
of ferrous ion was followed qualitatively, and
the determination of iron in it indicated the
presence of some iron in it’s ferrous state.
However, the presence of ferrous ion is rather difficult to attribute to its essential state in the enzyme and may be due to an artifact in isolation of iron from enzyme protein as also explained in the literature.\textsuperscript{12} An analogous situation has been observed in tyrosinase where the copper of oxidized protein is either entirely or partially in the reduced state.\textsuperscript{13,14} It has been reported by Kimura \textit{et al.}\textsuperscript{15} that the majority of iron is present in ferric state, hence the visible color of adrenodoxin may be due to ferric iron-protein chelation, and further, upon enzymatic reduction, valency of iron may change with simultaneous decolorization. The state of iron in this enzyme may also be assumed to be similar to this situation.

Our preparation of kojic acid oxidase was found to contain 0.5 \( \gamma \) of iron per mg protein, from which a minimum molecular weight of about 110,000 was calculated. Based on the molecular weight of 55,000 determined by TLC, the enzyme is considered to contain two atoms of iron in one molecule of the protein.

The results of inhibition by metal binding agents indicate that iron participates in the enzyme reaction. Low inhibition by \( o \)-phenanthroline, a strong trapping agent against ferrous iron, is probable due to the fact that the state of iron in the enzyme protein is ferric. If the binding state of iron in this enzyme is similar to that of ferredoxin, in treating with \( a-a' \) dipyridyl, iron may form an iron-chelate complex, and sulfide may form disulfide. This may probably cause a change in the structure of higher dimension on the enzyme.

This enzyme was inhibited by PCMB. Rabinowitz\textsuperscript{17} has reported that cysteine residue does not exist as free sulphydryl group since ferredoxin reacts with dithio-bis-2-nitrobenzene only in the presence of urea. In the present case it is possible that in the presence of PCMB, inorganic sulfur may react with PCMB, and iodoacetate, free sulphydryl group may not be present, since no inhibition occurs in addition of iodoacetate.

Hydrogen atom is released from kojic acid by enzyme reaction, and reduces ferric ion to ferrous, then the absorption peak at 408 m\( \mu \) disappears and a new absorption peak at 335 m\( \mu \) simultaneously appears with the formation of iron-protein complex by reduced enzyme and dehydrogenation product, comenic aldehyde. Blomstrom\textsuperscript{18} has been reported the model of iron ligand in ferredoxin of \textit{Clostridium pastorianum}. From similarity to ferredoxin, we would like to propose the model for kojic acid oxidase-comenic aldehyde complex shown in Fig. 16.

Kojic acid oxidase activity can be estimated from the absorbancy at 335 m\( \mu \) based on appearance of a characteristic absorption peak of this complex. As increase of the absorbancy at 335 m\( \mu \) is proportional to enzyme concentration (Figs. 9 and 10), it can be used for the determination of enzyme activity.

\textbf{Fig. 16. Proposed Model for Comenic Aldehyde-Iron Protein Complex.}


\textsuperscript{14} R. C. Kruger, \textit{Federation Proc.}, \textbf{18}, 267 (1957).


This method is fully available to determine the enzyme activity. In case of 5-methoxy kojic acid, which was not able to chelate with enzyme protein, enzyme activity was assayed by measuring oxygen uptake.

Under aerobic conditions, reduced enzyme was converted into oxidized enzyme by molecular oxygen, but under anaerobic conditions, hydrogen ion of reduced enzyme was transferred to NAD or thionine, which acted as hydrogen acceptor. Methylene blue and cresyl blue did not serve as hydrogen acceptor.

Although this enzyme has similar properties with bacterial and plant type of ferredoxin, it is likely that this enzyme acts not only as electron carrier like non-heme iron protein, but also catalyzes the dehydrogenation of kojic acid and the transfer of electron through iron contained in enzyme protein.

This enzyme belongs to the second type of simple protein according to Racker, and also belongs to multi enzyme complex, a new type iron protein, in view of function, which serves as both catalyst of dehydrogenation and electron transfer.

The reaction mechanism of this enzyme can be explained as shown in Fig. 16.