Short Communication

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Kojic Acid Oxidase

Sir:

In the course of earlier studies,^{1,2)} it has been shown that *Arthrobacter ureafaciens* strain (K-1) degraded kojic acid by the process, in which the intermediates of each stage were comenic aldehyde, comenic acid and uronic acids, respectively.

The present communication concerns with the kojic acid oxidizing enzyme which oxidizes kojic acid to comenic aldehyde.

The enzyme is a kind of iron protein, a new type of enzyme. The authors wish to propose the reaction mechanism of kojic acid oxidation as follows; The iron contained in the enzyme was reduced to ferrous state by the electron released from kojic acid accompanying with simultaneous oxidation of kojic acid to comenic aldehyde, and then the reduced enzyme was oxidized by oxygen of molecular state to form hydrogen peroxide.

Arthrobacter ureafaciens strain (K-1) was incubated by static culture at 30°C for 70 hr in a 500-ml flask containing 200 ml of the medium composed of 0.1% kojic acid, 0.1% NH_4NO_3 , 0.025% yeast extract and some of inorganic salts. The cells were harvested, washed twice with sterile water, suspended in $\text{m}/15$ potassium phosphate buffer (pH 7.8), and disrupted by sonic integrator for 15 min. Crude extracts were obtained by centrifugation for 10 min at $14,000 \times g$. The precipitate obtained by 20~40% ammonium sulfate saturation was dissolved in $\text{m}/20$ potassium phosphate buffer (pH 7.8). This fraction was applied to a Sephadex G-200 column (1.8×45.0 cm) equilibrated with

$\text{m}/20$ potassium phosphate buffer (pH 7.8), and 35 to 45 ml fraction was used as the enzyme solution.

FIG. 1. Absorption Spectra of Kojic Acid Oxidase.

1. Native kojic acid oxidase.
2. Reduced kojic acid oxidase with dithionite.
3. Oxidized kojic acid oxidase by aeration for 10 min after reduction.

The enzyme exhibits a strong absorption peak at 270 $\text{m}\mu$ and a characteristic peak at 408 $\text{m}\mu$. The latter band may be attributed to the enzyme-bound non-heme iron, because the enzyme has absorption spectrum of neither porphyrin nor flavin. When the enzyme was reduced with dithionite, an absorption peak at 408 $\text{m}\mu$ disappeared, and after aeration, an absorption spectrum of the oxidized form reappeared as shown in Fig. 1. Furthermore, the enzyme incubated with the substrate (kojic acid) showed an absorption spectrum similar to that of the enzyme reduced with dithionite. Thus reduced enzyme was restored

1) S. Nonomura and C. Tatsumi, *Bull. Agr. Chem. Soc. Japan*, **24**, 741 (1960).

2) J. Imose, S. Nonomura and C. Tatsumi, *Nippon Nogeikagaku Kaishi*, **41**, 94 (1967).

FIG. 2. Reduction of NAD.

Enzyme activity at start of incubation was 0.1367 (O.D.₃₃₅)/3 min/0.398 mg protein. After 60 min, remaining activity was 0.020, and after 63 min, 0.5 μ moles of NAD was added.

TABLE I. REDUCTION OF THIONINE LINKED TO OXIDATION OF KOJIC ACID BY THE KOJIC ACID OXIDASE UNDER THE ANAEROBIC CONDITION

Phosphate buffer (pH 7.8)	Kojic acid	Thionine	Enzyme	Reduction time
100 μ moles	5 μ moles	2 μ moles	1.0 ml	125 min
100	5	2	—	> 360
100	—	2	1.0	> 360

TABLE II. EFFECT OF METAL-BINDING AGENTS ON THE ACTIVITY OF KOJIC ACID OXIDASE

Metal-binding agent	Final conc. mmoles	Inhibition %
α - α' Dipyridyl	2	100.0
Benzoyl acetone	2	100.0
Citrate	2	93.0
Oxalate	2	90.8
<i>o</i> -Phenanthroline	2	36.0
8-Hydroxy quinoline	2	23.4
EDTA	10	83.0

to the oxidized form either by aeration or with NAD as shown Fig. 2. Under anaerobic condition, thionine could serve as an electron acceptor (Table I).

The enzyme showed an activity toward kojic acid, 5-methoxy kojic acid and benzyl

FIG. 3. Thin-layer Chromatogram of Kojic Acid Oxidase on Sephadex G-200.

Kojic acid oxidase (K), Chymotrypsinogen (Chy), Horseradish peroxidase (Per), Bovine serum albumin (A) and Human γ -globulin (γ -G).

kojic acid. The optimal pH was 7.8 in the reaction mixture containing 3 μ moles of kojic acid, 100 μ moles of potassium phosphate buffer (pH 7.8), and 0.5 ml of the enzyme solution in a total volume of 3.0 ml. Neither NAD nor NADP was required for the enzyme activity. The enzyme was inhibited, much or less, by various metal-binding agents (Table II).

The iron content of the enzyme in the fraction eluted from the Sephadex G-200 column was estimated to be 0.5 γ per mg enzyme protein by the *o*-phenanthroline method, and molecular weight of the enzyme was assumed to be 55,000 by thin layer chromatography method using Sephadex G-200 (Fig. 3). From the above results, the enzyme contains 2 atoms of iron per molecule.

FIG. 4. A New Absorption Peak of the Kojic Acid Oxidase Appeared at 335 $m\mu$ after Incubation with Kojic Acid.

FIG. 5. Ultraviolet Spectra of Semicarbazones of 5-Methoxy Comenic Aldehyde and the Reaction Product.

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

When the enzyme was incubated with the substrate (kojic acid), a new absorption peak was observed at 335 $m\mu$ (Fig. 4). This absorption peak might result from the formation of the complex between kojic acid and iron

of ferrous state. If so, the enzyme activity can be estimated from the intensity at 335 $m\mu$ absorption.

The reaction product of the enzyme and 5-methoxy kojic acid was identified as 5-methoxy comenic aldehyde by its conversion to the corresponding semicarbazone and by comparison of their ultraviolet spectra (Fig. 5).

From the above results, it is summarized that (1) the molecular weight of the enzyme is about 55,000; (2) the enzyme contains 2 atoms of iron per molecule; (3) the iron atom may participate in transport of the electron released by dehydrogenation of kojic acid, and (4) the reaction products are comenic aldehyde and hydrogen peroxide.

We wish to propose the model for the reaction mechanism as follows.

Detailed description of this study will be presented in this journal.

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