Note

Inhibition Study of Tyrosinase
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Tyrosinase (monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing monooxygenase catalyzing both *o*-hydroxylation of monophenols and oxidation of *o*-diphenols to *o*-quinones.¹⁾ Lerch²⁾ established, through work on *Neurospora crassa* tyrosinase, that it contains two copper ions per active subunits. However, the biochemical characterization of the reactions involved in the synthesis of melanin from tyrosine by tyrosinase has not yet been completed.

Our previous paper described the isolation of three molecular forms (I~III) of protyrosinase and catalytically active tyrosinases (I~III) from fresh mycelia of *Aspergillus oryzae* RIB-128.³⁾ This study was undertaken to describe the inactivation kinetics by mimosine, tropolone, kojic acid, and benzoic acid using *Aspergillus oryzae* tyrosinase I as the enzyme source.

Kojic acid (Lot M5B 4023) and diethyldithiocarbamate (Lot V5A 9293) were purchased from Nacalai Tesque, Inc. Benzoic acid (Lot 142752) was obtained from Kokusan Chemical Co. *p*-Aminobenzamidine hydrochloride (Lot FC 101), 3-monoiodotyrosine (Lot A-101), 3,5-diiodotyrosine (Lot AW 01), tyroxine (Lot AP02), α -methyl-L-DOPA (Lot AW 01), 5-hydroxytryptophan (Lot AN01), and protocatechuic acid (Lot AW 01) were from Tokyo Kasei Co. L-Mimosine (Lot 112F-8110), tropolone (Lot 45F-0180), D-tyrosine (Lot 94F-0384), and D-DOPA (Lot 104C-01941) were from Sigma, St. Louis, MO, USA. α -Methyl-DL-tyrosine methylester was from Research Organic Co., Cleveland, Ohio, USA.

Purification of protyrosinase was done by the method in a previous paper.³⁾ The major band of protyrosinase I was

extracted from polyacrylamide gel electrophoresis with 0.5 M phosphate buffer, pH 6.0, by the method of Kobayashi *et al.*⁴⁾

The protyrosinase I was activated by incubation with an acidic buffer at pH 3.0 and 0°C for 30 min, as in our previous paper.³⁾ The reaction mixture consisted of 200 μ l of 0.2 M sodium acetate-HCl buffer at pH 3.0 and 50 μ l of protyrosinase solution. After 30 min at 0°C the activation reaction was stopped by adding 3 ml of 0.5 M phosphate buffer, pH 6.0. The activated tyrosinase I was homogeneous by polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis⁵⁾ was done using 7.5% polyacrylamide gel in Tris-glycine buffer pH 9.4. The protein was stained with Coomassie brilliant blue G-250.

For tyrosinase measurement, 2 ml of 25 mM L-DOPA solution was added to 3 ml of the activated tyrosinase solution described above. After 5 min at 30°C, tyrosinase activity in the reaction mixture was measured spectrophotometrically at 475 nm in a Shimadzu model UV 200 spectrophotometer. One enzyme unit (katal) was defined as the amount transforming 1 mol of L-DOPA per sec at 30°C and pH 6.0.

The values of K_m and k_{cat} were found graphically from Lineweaver-Burk plots. The K_i value was calculated from Dixon plots.⁶⁾

For L-DOPA oxidation at pH 6.0 with the tyrosinase I from *Aspergillus oryzae*, a K_m value of 2.3 mM, a k_{cat} value of 4,500 sec⁻¹, and a k_{cat}/K_m value of 1,957 sec⁻¹ mM⁻¹ were calculated.

Ohba *et al.*⁷⁾ identified L-DOPA and protocatechuic acid as main precursors of the pigment of brown-rice koji. Haneda *et al.*⁸⁾ reported that L-DOPA can be synthesized effectively from L-tyrosine under acidic conditions (pH 2 to 5) by *Aspergillus oryzae*.

Effects of various reagents on the oxidase activity of tyrosinase I from *Aspergillus oryzae* at pH 6.0 were observed. Metal-chelating reagents, diethyldithiocarbamate (DDC) and tropolone, were effective inhibitors of the tyrosinase from *Aspergillus oryzae*, suggesting that the tyrosinase is a metal-containing enzyme. Since this compound has been known to bind with Cu²⁺ at the active site of *Vibrio* tyrosinase,⁹⁾ the same mechanism of inhibition is suggested. But ethylenediamine tetraacetate (EDTA) and *o*-phenantroline had no inhibitory effect on the tyrosinase activity.

The tyrosinase was strongly inhibited by substrate analogues, kojic acid, mimosine, and benzoic acid. Protocatechuic acid, tryptophan, and 5-hydroxytryptophan were weak inhibitors for the tyrosinase. D-DOPA, α -methyl-L-DOPA, 3-monoiodotyrosine, 3,5-diiodotyrosine, α -methyltyrosine methylester, and thyroxine had no inhibitory action.

A dixon plot of the tyrosinase with mimosine as an

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inhibitor showed a competitive inhibition. The value of the inhibitor constant, K_i , for mimosine was $0.13 \mu\text{M}$. Competitive inhibitions were also observed with tropolone, koji acid, and benzoic acid, and the K_i values were $0.22 \mu\text{M}$, $12 \mu\text{M}$, and $700 \mu\text{M}$, respectively.

Among the substrate-analogues mimosine, kojic acid, and benzoic acid, mimosine was the strongest inhibitor for the tyrosinase. Mimosine is an amino acid analogue isolated from certain plants belonging to the two legume genera, *Mimosa* and *Leucaena*. It is structurally related to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and is toxic to various animals.¹⁰⁾

In comparison with the kinetic parameters of the tyrosinases, the tyrosinase I from *Aspergillus oryzae* had about 3-fold larger value of K_m , and a 14-fold higher value of k_{cat} than those of *Neurospora crassa* tyrosinase.¹¹⁾ The k_{cat}/K_m value of the tyrosinase from *Aspergillus oryzae*³⁾ was about 4.3-fold higher than that of *Neurospora crassa* tyrosinase and about 2.3-fold higher than that of mushroom tyrosinase.¹²⁾

Gutteridge and Robb¹¹⁾ showed with *Neurospora* tyrosinase that the nature of the inhibition of benzoic acid was competitive with respect to *o*-diphenol, but that it was a mixed inhibitor of oxygen. Cyanide, which behaved as a reversible inhibitor under the assay conditions, was found to be a competitive inhibitor of oxygen and a noncompetitive inhibitor of *o*-diphenol.

The nature of the inhibition exerted by benzoic acid on mushroom tyrosinase was reported by Duckworth and Coleman.¹²⁾ Benzoic acid was competitive with catechol. A K_i value of $1.02 \pm 0.17 \mu\text{M}$ was found. They suggested that two separate substrate-binding sites exist within the active center, one for the catechol site and one for oxygen. Benzoic acid competes for the catechol site, while cyanide interacts with the oxygen site.¹²⁾

Saruno *et al.*¹³⁾ mentioned that exposure of a tyrosinase from mushroom to a high concentration of kojic acid resulted in loss of activity.

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