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Effect of UV Light on Biological Activity of Tyrosinase in Buffer Solution

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It has been reported previously that the UV irradiation of mushroom tyrosinase resulted in a logarithmic loss of biological activity due to the conformational changes in the enzyme molecule. The purpose of the present study was to investigate the factors influencing the radiosensitivity of enzymatic activity. A similar rate of the catalytic activity loss at different pH supported the attribution to the conformational change of the enzyme molecule but not to the active site damage. The presence of potassium chloride and the absence of oxygen resulted in only a little protection against enzyme photoinactivation. Activity survival curve as a function of radiation dose at a higher enzyme concentration showed to have a shoulder which indicate the mutual protection of the enzyme molecules. Copper (II) protected the loss in catalytic activity of enzyme on irradiation. This was explained in terms of scavenging of the hydrated electron by copper (II). Therefore, it was concluded that photoinactivation of this enzyme was mainly due to conformational changes caused by the damage of constitutional aromatic amino acid residues but also partially due to inactivation of copper of enzyme with hydrated electron.

INTRODUCTION

Photoinactivation of many enzymes has been studied. There are two types of damage in enzyme molecules by irradiation, one is the damage of active site of the enzyme molecules[1-3] and the other is the some constitutional amino acid residues damage[4-7] which are responsible for the secondary and tertiary structure of enzyme. The enzyme tyrosinase (*o*-diphenol: O_2 oxidoreductase, EC 1.10.3.1) catalyses the aerobic oxidation of various mono- and diphenols to produce the pigment melanin.[8] Hyperpigmentation of the skin of mammals as the result of irradiation is a well known phenomenon.[9,10] However, the mechanisms which regulate this radiation-induced melanogenesis are unknown. Only a few irradiation studies weredone using purified tyrosinase.[11,12] Our previous studies[12] ruled out the possibility of direct

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ultraviolet activation of tyrosinase and rather showed that the enzyme molecules were inactivated with conformational changes. The present studies are undertaken to investigate the various factors influencing the radiosensivity of enzyme activity.

MATERIALS AND METHODS

Tyrosinase solutions were prepared by dissolving lyophilized, salt free mushroom tyrosinase (Sigma Chemical Company, U. S. A.) in 0.1 M phosphate buffer, pH 6.5. The enzyme appeared essentially homogeneous on Sephadex gel filtration and polyacrylamide gel electrophoresis and used without further purification. Bi-distilled water was used throughout these studies.

An high pressure mercury vapour lamp (main wavelength 2537 Å) was used as the source of ultraviolet light. The dose rate was 2.5×10^8 ergs/cm^2/min, measured with potassium ferrioxalate actinometer.[13] Tyrosinase solutions kept in a specially devised containers[14] were irradiated in the presence and absence of air by ultraviolet. The temprature during irradiation was maintained at 25 ± 0.5°C with the help of thermostat unless otherwise mentioned.

The enzyme activity was measured separately towards catechol, 3-(3.4-dihydroxyphenyl) DL-alanine (dopa), chlorogenic acid, tyrosine, and tyramine according to the method previously reported.[12] The enzymatic activity towards hydroquinone, *p*-cresol, and *m*-cresol was measured with a similar method as used for catechol.

For fluorescence measurements, an Aminco-Bowman spectrophotofluorometer with fused quartz cuvettes of 1.3 cm path length was used. The fluorescence of tryptophyl residues in tyrosinase was monitored at the emission maximum 350 nm by exciting the samples with 286 nm of light.

The apparent rate constants, K, for enzymatic activity were calculated from the slope of the straight line obtained by the plot of $-\log(y_\alpha - y_t)$ versus radiation dose.[15]

RESULTS AND DISCUSSION

The ultraviolet irradiation of tyrosinase in buffer solutions resulted in a logarithmic loss of biological activity. Figure 1 shows the catalytic activity and fluorescence intensity remaining after irradiation. The loss of catalytic activity in uv irradiated enzyme molecules can be due to either "all-or-none"-type loss of activity or "partial modification" of activity due to modified molecules which are produced as the result of irradiation. If the "all-or-none"-type of mechanism is operative, there should be no change in the Michaelis-Menten constant. Our kinetics studies showed the changes of K_m values by uv irradiation of mushroom tyrosinase,[12] which clearly indicate that the "partially modified" enzyme molecules are produced as the result of irradiation. Adelstein and Mee[16] has reported the similar phenomenon for glutamate dehydrogenase and Winstead and Reece[17] for lactate dehydrogenase. The fluorescence intensity of tyrosinase was found to decrease with increasing radiation dose (Fig. 1), though the decreasing rate was less than that of the catalytic activity.

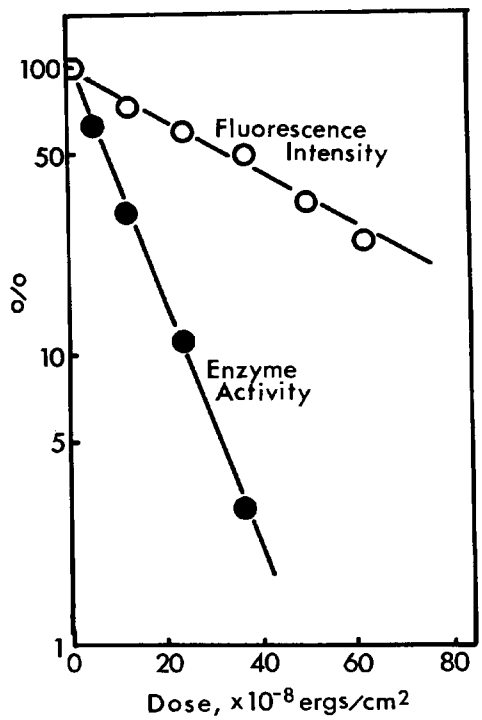

Figure 1. Effect of UV irradiation on tyrosinase activity and fluorescence intensity. Enzyme was irradiated at a concentration of 0.2 mg/ml in 0.1 M phosphate buffer at pH 6.5 and 25°C in air. Fluorescence intensity was measured at 350 nm by exciting the samples at 286 nm. Enzymatic activity was measured using 8 mM dopa at pH 6.5 and 37°C.

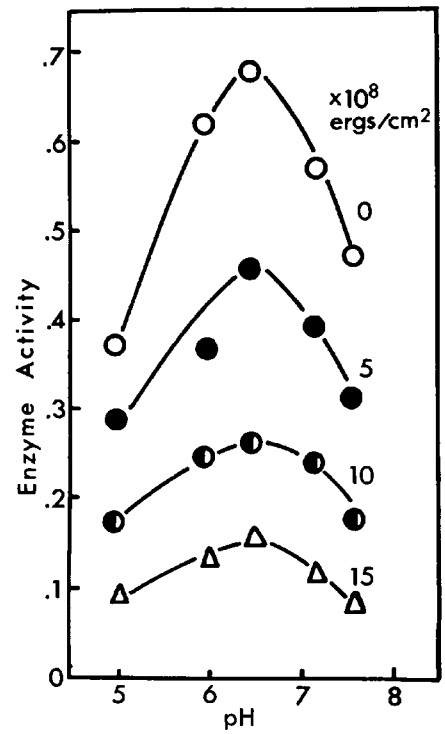

Figure 2. Effect of pH on the photoinactivation of tyrosinase. Enzyme was irradiated at a concentration of 0.2 mg/ml in 0.1 M phosphate buffer at different pH and 25°C in air. Enzymatic activity was measured using 8 mM dopa at pH 6.5 and 37°C.

The reason for this decrease in fluorescence intensity on uv irradiation may be due to the damage of amino acid residues such as tryptophan and tyrosine which mainly absorb photons from used uv lamp. For this enzyme, the amino acid residues present at the active site have not been identified. The involvement of tryptophyl residue at the active site has been ruled out in our previous report.[12] Therefore, such aromatic amino acid residues which are not at the active site may result in conformational changes in the enzyme molecules which decreases the enzymatic activity.

Tyrosinase has been shown to be active towards various mono- and diphenols.[12,18-20] The catalytic activity of irradiated and nonirradiated enzyme towards these substrates was measured and the results are given in Table 1. For diphenols, the per cent activity was expressed with respect to catechol and for monophenols with

respect to *p*-cresol. The native enzyme showed high activity towards catecnol and dopa and was observed to be the same, for chlorogenic acid it is only twenty per cent and practically no activity towards hydroquinone. The enzyme was found to be active with *p*-cresol followed by *m*-cresol, tyrosine and tyramine. These difference of reaction yield by substrate without irradiation could be due to the different abilities of the enzyme to recoganize the substrates. If the radiation induced damage of the enzyme is mainly "all-or-none"-type loss of activity, the decreasing rate of the reaction yields of the different substrates with enzyme should be the same to each other by irradiation. However, the loss of catalytic activity after irradiation was found to follow the different pattern for the substrates (Table 1). This also supports the formation of the "partially modified" enzyme molecules.

When the tyrosinase was irradiated at various pH's and the activity was measured using dopa as substrate, the maximum activity was observed at pH 6.5 not only for controls but also for irradiated ones (Fig. 2). The progress of inactivation was followed, as the dose increased, it was observed that the biological activity decreased to 22 per cent of the initial activity at a dose of 15.0×10^8 ergs/cm.^{2} Due to irradiation there is overall loss in catalytic activity at all pH values indicating that the changes in the conformation induced by uv irradiation are independent of pH. No change in optimum pH after irradiation might suggest that the amion acid residues

Table 1. The Effect of UV Light on Tyrosinase Activity Towards Various Mono- and Diphenols.

Substrate	Relative Activity, (%)				
	0 ergs/cm^2	5.0×10^8 ergs/cm^2	10.0×10^8 ergs/cm^2	15.0×10^8 ergs/cm^2	20.0×10^8 ergs/cm^2
Diphenols					
Catechol	100.0	90.4	75.0	52.7	28.7
Dihydroxy-phenyl-alanine (dopa)	101.4	70.2	43.9	24.4	11.4
Chlorogenic acid	20.8	13.8	9.0	3.3	—
Hydroquinone	1.4	0.75	0.55	0.55	—
Monophenols:					
p-Cresol	100.0	71.5	40.6	20.4	6.4
m-Cresol	90.9	60.2	30.9	15.4	3.3
Tyrosine	76.3	34.4	9.8	1.9	—
Tyramine	31.8	16.2	6.8	2.7	—

The concentration of various substrates used were: dopa, 8 mM; 10 mM each of catechol, hydroquinone, *p*-cresol, and *m*-cresol; and 2 mM each of chlorogenic acid, tyrosine and tyramine. The catalytic activity was measured in 0.1 M phosphate buffer, pH 6.5 at 37°C. Experimental details are given in the text. The per cent activity for diphenols was expressed with respect to catechol and for monophenols, with respect to *p*-cresol.

at the active site are mostly not modified upto a dose of 15.0×10^8 ergs/cm.^{2} The effct of pH on the photosensitivity of ribonuclease[21] and collagenase[22] has shown that the inactivation of enzyme was more pronounced at alkaline pH and little inactivation at pH less than 5.0, showing the involvement of histidine residue at the active site of enzyme. However, in our case, only a small change of relative velocity was observed at different pH (Fig. 3). This indicates that photoinactivation of tyrosinase upto the dose of 15.0×10^8 ergs/cm^2 was mainly caused by the "partial modification" of enzyme molecules due to the conformational changes.

The dose effect curve were determined at various enzyme concentrations at pH 6.5 in 0.1 M phosphate buffer (Fig. 4). The loss of catalytic activity was found to be dependent on enzyme concentration. A rapid linear loss in biological activity at lower concentration as a function of irradiation dose was observed, whereas at higher concentration there was a shoulder in the small dose area. At higher concentration, relatively less number of enzyme molecules may be affected on irradiation according to the protective effect of enzyme molecules due to their increased population; the irradiation effect towards the molecules are minimized on account of intermolecular interactions. This possibility could be more realistic as *in vivo* higher irradiation dose is needed to bring about a significant change in a variety of biolo-

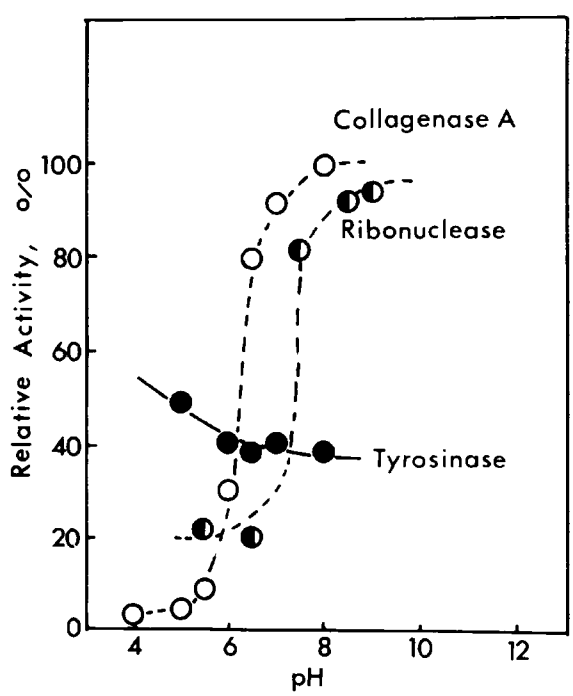

Figure 3. Effect of pH on the photoinactivation of tyrosinase (10.0 $\times10^8$ ergs/cm^2) compared to those of ribonuclease[21] and collagenase[22]. The rate of photoinactivation is expressed as the percentage.

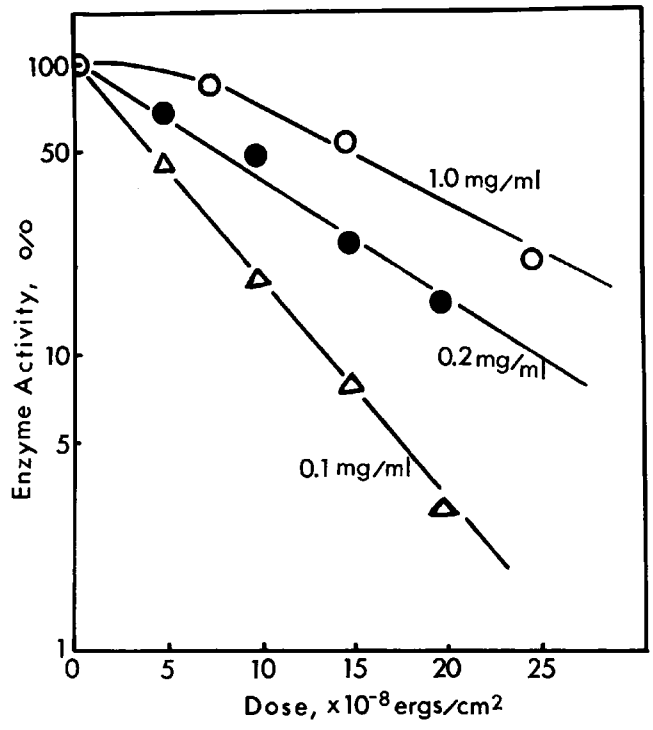

Figure 4. Dose-effect curve at various enzyme concentration. Enzyme was irradiated in 0.1 M phosphate buffer at pH 6.5 and 25°C in air. Enzymatic activity was measured using 8 mM dopa at pH 6.5 and 37°C.

gical systems.

The rate of loss of biological activity of tyrosinase was a little lowered in the presence of 0.1 M potassium chloride when compared to the enzyme irradiated in the absence of it (Fig. 5). For fifty per cent loss in enzyme activity in the presence and absence of potassium chloride, the irradiation dose of 8.7×10^{3} ergs/cm^2 and 6.2×10^{3} ergs/cm^2 respectively were needed. The protection may be ascribed to a little increased stability of subunit structure in the presence of salt which might protect the unfolding of enzyme molecules. Similarly, O_2-free system showed a little decrease of catalytic activity loss compared to air system, though the figure is not shown here. It indicates that O_2 effect is very low on this photoinactivation.

Copper (II) has been reported to protect catalase,[23] trypsin and bovine serum albumin[24,25] against photoinactivation. The effect of the addition of copper ions on the uv inactivation of tyrosinase was investigated. Copper sulphate at a concentration of 1.0×10^{-4} M was used as the source of copper ions. Although the addition of copper ions did not affect the enzymatic activity without irradiation. It was found to protect the catalytic activity on irradiation (Fig. 6). The apparent rate constant, K, of inactivation was decreased from 22.7×10^{-2} min^{-1} to 12.85×10^{-2} min^{-1}

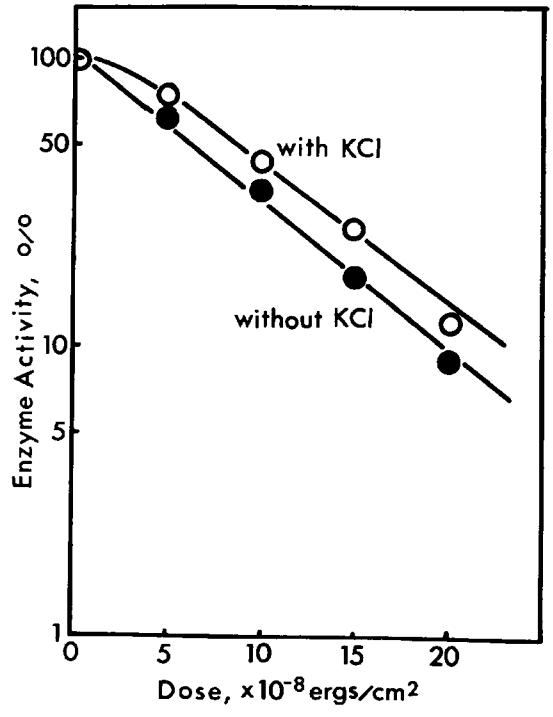

Figure 5. Effect of potassium chloride on the photoinactivation of tyrosinase. Enzyme was irradiated at 0.2 mg/ml in 0.1 M phosphate buffer in the presence of 0.1 M potassium chloride at pH 6.5 and 25°C in air. Enzymatic activity was measured using 8 mM dopa at pH 6.5 and 37°C.

in the presence of copper ions. Only a slight protective effect of the other metal ions such as Fe^{+++} and Zn^{++} was observed.

Our recent studies (unpublished) showed that the protective effect of copper ions on catalytic activity of tyrosinase when irradiated with gamma-rays is higher than that with uv light. The primary species induced inactivation of enzymatlc activity of tyrosinase in the case of gamma-ray irradiation was hydrated electron but not OH radical as will be reported in a subsequent paper. Production of hydrated electron is also known in uv irradiation.[26] Therefore, there should be a part of contribution of hydrated electron to tyrosinase photoinactivation.

The mechanism of substrate oxidation by this enzyme has been given by Mason[27] as follows:

$$\text{Protein-Cu}_2^{+} + \text{O}_2 \longrightarrow \text{Protein-Cu}_2\text{-O}_2 \quad (1)$$

$$\text{Protein-Cu}_2\text{-O}_2 + \text{monophenol} + 2\text{H}^{+} \longrightarrow \text{Protein-Cu}_2^{++} + o\text{-diphenol} + \text{H}_2\text{O} \quad (2)$$

$$\text{Protein-Cu}_2^{++} + 2\text{e}^{-} \longrightarrow \text{Protein-Cu}_2^{+} \quad (3)$$

$$\text{Protein-Cu}_2^{++} + o\text{-diphenol} \longrightarrow \text{Protein-Cu}_2^{+} + o\text{-quinone} + 2\text{H}^{+} \quad (4)$$

Hydrated electron may react with Protein-Cu_2^{++} according to reaction (5).

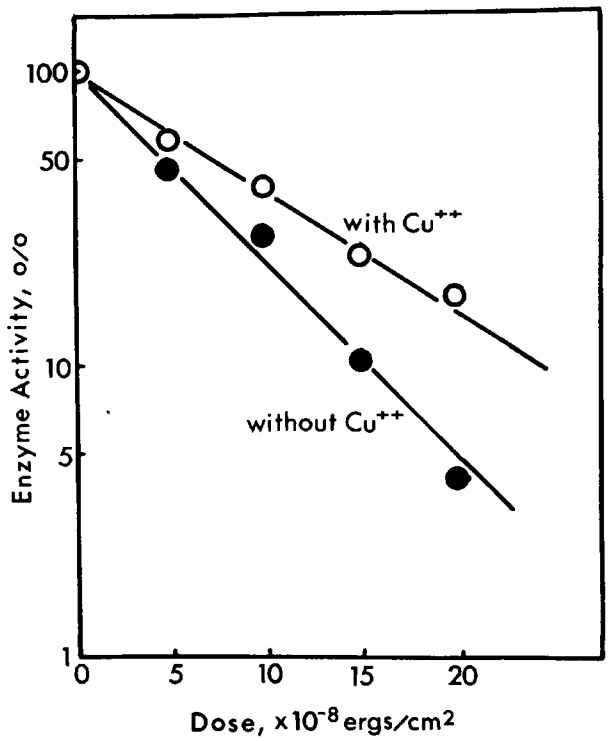

Figure 6. Effect of copper ions on the photoinactivation of tyrosinase. Enzyme was irradiated at 0.2 mg/ml in 0.1 M phosphate buffer in the presence of 1.0×10^{-4} M copper sulphate at pH 6.5 and 25°C in air. Enzymatic activity was measured using 8 mM dopa at pH 6.5 and 37°C.

$$\text{Protein-Cu}_2^{++}+4e_{aq}^- \longrightarrow \text{Protein-Cu}_2+4H_2O \qquad (5)$$

Protein-Cu_2 is no longer able to catalyse the aerobic oxidation of monophenol and diphenol. In Cu^{++} added solution, a reaction $Cu^{++}+2e_{aq}^- \longrightarrow Cu+2H_2O$, can compete with reaction (5). The contribution of hydrated electron to total photoinactivation would be about twenty four per cent as estimated from the results of Fig. 6. The protective nature of Cu^{++} much higher than those of Fe^{+++} and Zn^{++} would be due to the higher reaction rate of Cu^{++} with hydrated electron. Indeed, the reaction of Cu^{++} with hydrated electron precedes that of Fe^{+++} as known in Fricke dosimeter system.[28]

Furthermore, the addition of sulfhydryl compounds such as 2-mercaptoethanol, glutathione reduced and ascorbic acid (2.0×10^{-3} M) to the reaction system resuted in almost complete inhibition of enzymatic activity before and after irradiation. These agents might act to inhibit the tyrosinase catalysed oxidation by their reducing reactivity.

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