Hydrated Electron-Induced Inactivation of Tyrosinase in Aqueous Solution by Exposure to Cobalt-60 Gamma-Rays

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Publication of the Thesis

(1) Hydrated-Induced Inactivation of Tyrosinase in Aqueous Solution by Exposure to Cobalt-60 Gamma-Rays. I. Cresolase Activity.

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(2) Hydrated-Induced Inactivation of Tyrosinase in Aqueous Solution by Exposure to Cobalt-60 Gamma-Rays. II. Catecholase Activity.

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CONTENTS

GENERAL INTRODUCTION

Ionizing radiation attacks organisms randomly and injures various molecules in the cells. For long time DNA has been thought to be a primary target on radiation-induced injuries. However the radiation-induced damages of other cellular components such as membranes and proteins including enzymes, must be important for cell death.[1)]

The radiation effects on protein molecules are 1. degradation of amino acid residues, 2. breakage of peptide bonds, 3. dissociation of hydrogen bonds, 4. incision of disulfide bonds and 5. production of cross linking in molecules.[2)] All of these events cause conformational change, fragmentation and aggregation, or raise proteolytic susceptibility in cellular condition.[2, 3)] However, the generalization of radiation effects on proteins seem to be difficult, because proteins have different construction, different activity and various mechanisms of activity control.[2)]

In the present study, the author subjected the radiation effect on tyrosinase. Tyrosinase (EC 1. 12. 18. 1) is a copper-containing protein, which is one of well-known metalloenzymes.[4-10)] The present study is to clarify what the main active species inactivates tyrosinase. Cells contain much amount of H_2O. The weight of H_2O occupies about 70 % of whole living cells. Therefore, main effects induced by radiation must be considered as indirect events with some kinds of active species derived from H_2O. It is well known that the main radiolytic active species are

hydroxyl radicals (·OH: G = 2.7) and hydrated electrons (e^-_{aq}: G = 2.6).[11, 12] Other species such as hydrogen atoms (·H: G = 0.6) and hydrogen peroxides (H_2O_2: G = 0.7) are low yields.[11, 12]

Previously many investigators studied the radiation-induced inactivation of enzymes.[2] All of them presented that the main active species which inactivates enzymes was ·OH. However, there is no studies on the focus into radiation-induced inactivation of metalloproteins. Then the author tried to study on the radiolytic inactivation of tyrosinase. Tyrosinase has two enzymatic activities. One is monophenolmonooxygenase activity (cresolase activity) and other is *o*-diphenoloxidase activity (catecholase activity).[4-10] Which of ·OH and e^-_{aq} is the main species to cause inactivation of this enzyme? The present study is a comparison of radiation effect on both enzymatic activities. It is interesting whether the inactivation mechanism is the same or not for these two activities of tyrosinase.

Tyrosinase participates in the metabolic pathways of catechol and catecholamine.[4-10] These pathways are related with the melanin production in animal skins and plant tissues. These essential enzyme activities seem to protect some organisms against irradiation. In addition, ultraviolet (UV) is also able to cause H_2O-derived radicals.[13] Sun light containing UV raises amount of H_2O-derived active species in human skin cells. Melanin is a protector against UV in human skin. Therefore, tyrosinase that produces melanin, seems to be important to protect

against UV. From this point of view, inactivation yields of both activities of tyrosinase are compared with those of other enzymes reported.

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CHAPTER 1

HYDRATED-INDUCED INACTIVATION OF TYROSINASE IN AQUEOUS SOLUTION BY EXPOSURE TO COBALT-60 GAMMA-RAYS. I. CRESOLASE ACTIVITY

ABSTRACT

Tyrosinase (0.2 mg/ml of 0.1 M phosphate buffer solution, *pH* 6.5) which has cresolase and catecholase activities was irradiated with ^{60}Co gamma-rays. The cresolase activity was measured at varying radiation doses under various atmospheric conditions. D_0 was found to be 350 Gy in N_2-saturated solution. The hydroxyl (OH) radical has been shown to be main species involved in radiation-induced inactivation of most of enzymes. However, OH radical scavengers, *t*-BuOH and MeOH, had no effect in this study. In addition, O_2 that acts generally as an enhancer of OH radical-induced inactivation also had little effect. On the other hand, N_2O as a hydrated electron (e^-_{aq}) scavenger, and Cu^{++} markedly inhibited the inactivation. These results indicate that e^-_{aq} is the main species involved in inactivating the cresolase activity, reducing Cu^{++} as the active center.

INTRODUCTION

Tyrosinase (EC 1. 12. 18. 1) is a copper-containing protein that has two enzymatic activities, monophenolmonooxygenase activity (cresolase activity) and *o*-diphenoloxidase activity (catecholase activity). The enzymes are widely distributed in a lot of organisms from microbials, plants and animals. It has been reported that copper contained in this enzyme is the essential moiety of the active center.[1-7] The present report deals with the effect of ionizing radiation on tyrosinase in relation to the amino acid synthesis in cells and melanin production in human skin.

In radiation biology, one of main objects is to clarify radiation-induced effects on molecules constructing organisms such as DNA, proteins (including enzymes) and others. When the aqueous systems are exposed to ionizing radiation, two main active species are produced; OH radicals and e^-_{aq} derived from water molecules.[8] It is known that enzymes are inactivated mainly by OH radicals but not by e^-_{aq}.[9] In addition, Cu^{++} has been also shown to be a sensitizer for the radiation-induced inactivation of enzymes.[10, 11]

The purpose of this study was to determine whether the radiation-induced inactivation of tyrosinase takes place in the same manner of most of proteins which are inactivated mainly by OH radicals. As the first step, the cresolase activity was studied.

MATERIALS AND METHODS

Materials:

Mushroom tyrosinase (lyophilized, salt-free, grade III, electrophoretically pure) was purchased from Sigma Chemical Company (U. S. A.). L-Tyrosine and tyramine were from Wako Pure Chemical Industries (Japan). Copper compounds used; cupric sulfate and chloride, and cuprous chloride were from Wako Pure Chemical Industries (Japan). All the chemicals used were of analytical grade. Triply distilled water was used throughout these studies. N_2O (Showa Denko, Japan), N_2 and O_2 (Nihon Sanso, Japan) gases used were at high grade (>99.99 %).

Irradiation:

Tyrosinase solutions were prepared in 0.1 M phosphate buffer, *pH* 6.5, to a final concentration of 1.56×10^{-6} M (0.2 mg/ml). The solutions were saturated with N_2, O_2 and N_2O gases by bubbling for 15 min, respectively, prior to gamma-ray irradiation, and *t*-BuOH (10^{-3} M and 10^{-2} M final concentrations), MeOH (10^{-3} M and 10^{-2} M final concentrations), and copper compounds (at different concentrations) were also added to the solutions. Tyrosinase solutions (1 ml) in Pyrex glass tubes (Ø 1.2 cm x 9 cm) were irradiated with ^{60}Co gamma-rays (Shimadzu RT-10000S) at a dose rate of 200 Gy/min under various atmospheric conditions at 0 °C. Dosimetry was performed with a Fricke dosimeter.[12)]

Enzyme Assay:

The cresolase activity of tyrosinase was measured using tyrosine and tyramine as substrates according to the method reported previously.[13] The reaction mixture consisted of 2 ml of substrate solution (2 mM). 1.8 ml of 0.1 M phosphate buffer, pH 6.5, and 0.2 ml of the enzyme solution. After incubation of the mixture at 37 °C for 5 min, the absorbance of dopachrome produced was measured at 480 nm by Hitachi 124 spectrophotometer. The data presented here are averages of not less than three individual determinations.

RESULTS

Tyrosinase was exposed to gamma-rays in aqueous solution under N_2 and the cresolase activity was measured using tyrosine as a monophenol substrate (Fig. 1). The enzymatic activity decreased in an exponential manner. D_0 (37 % survival dose) value was found to be 350 Gy. The survival curve with tyramine used as another monophenol substrate was almost the same to that with tyrosine (data not shown).

No effect of gamma-ray irradiation on Michaelis-Menten constant, K_m, was found for cresolase activity with indicating no lowering of affinity with the irradiated enzyme as shown in Fig. 2. The experiment with tyramine used as another monophenol substrate was almost the same to that with tyrosine (data not shown).

Effects of O_2, *t*-BuOH, MeOH and N_2O on the inactivation of the enzyme activity induced by radiation are also shown in Fig. 1. Oxygen showed little enhancement of the inactivation of the enzyme, and no effect of *t*-BuOH and MeOH, both OH radical scavengers ($(CH_3)_3COH + \cdot OH \rightarrow \cdot CH_2(CH_3)_2COH + H_2O$, $CH_3OH + \cdot OH \rightarrow \cdot CH_2OH + H_2O$), were also found ; that is , these alcohols never protected the enzyme against inactivation. However, the presence of N_2O that is an e^-_{aq} scavenger ($N_2O + e^-_{aq} \rightarrow \cdot OH + OH^- + N_2$) markedly blocked the inactivation. Therefore, the main species involved in the inactivation of this enzymatic activity must be e^-_{aq} but not OH radicals.

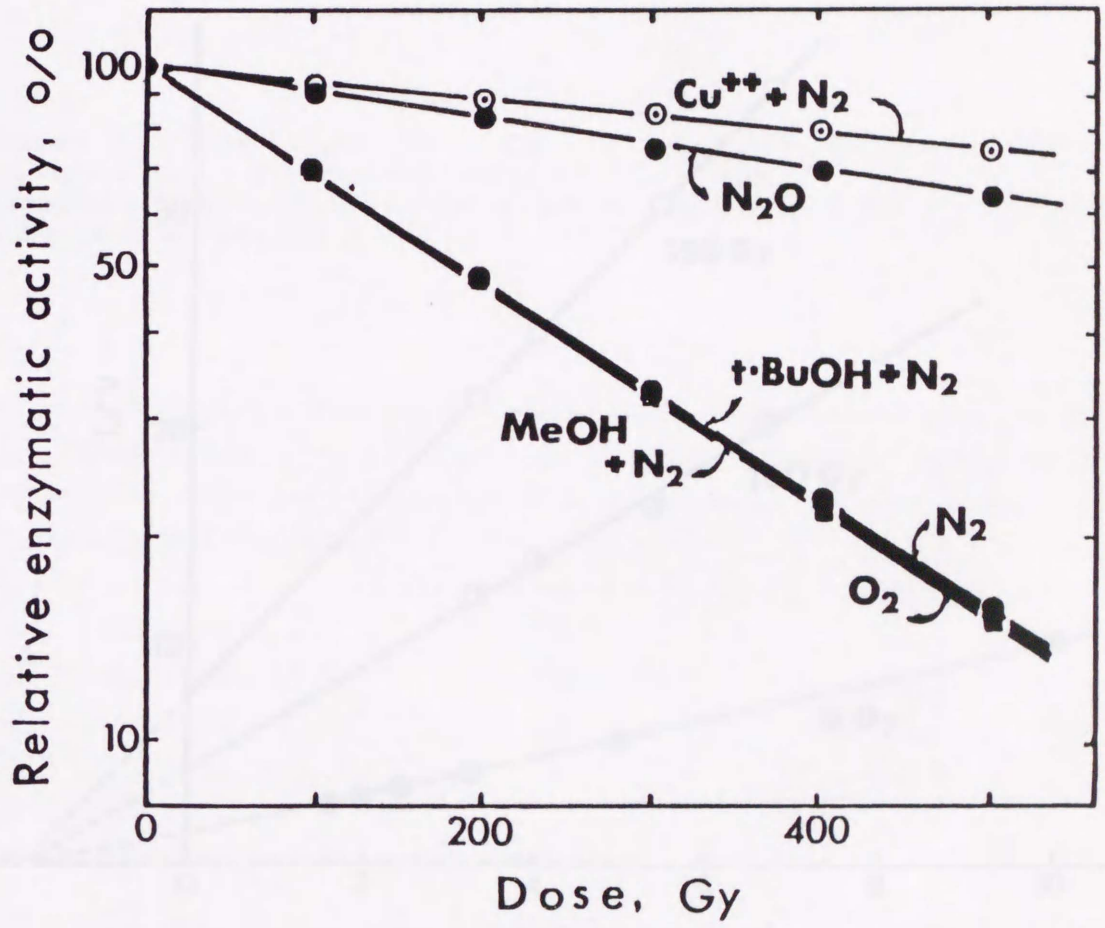

Fig. 1. Survival curve of cresolase activity of tyrosinase in N_2-saturated solution (0.2 mg/ml of phosphate buffer, pH 6.5) irradiated with ^{60}Co gamma-rays, and the effects of presence of MeOH (10^{-2} M), t-BuOH (10^{-2} M), O_2 (saturated), N_2O (saturated) and Cu^{++} (10^{-4} M). SE: <2 %.

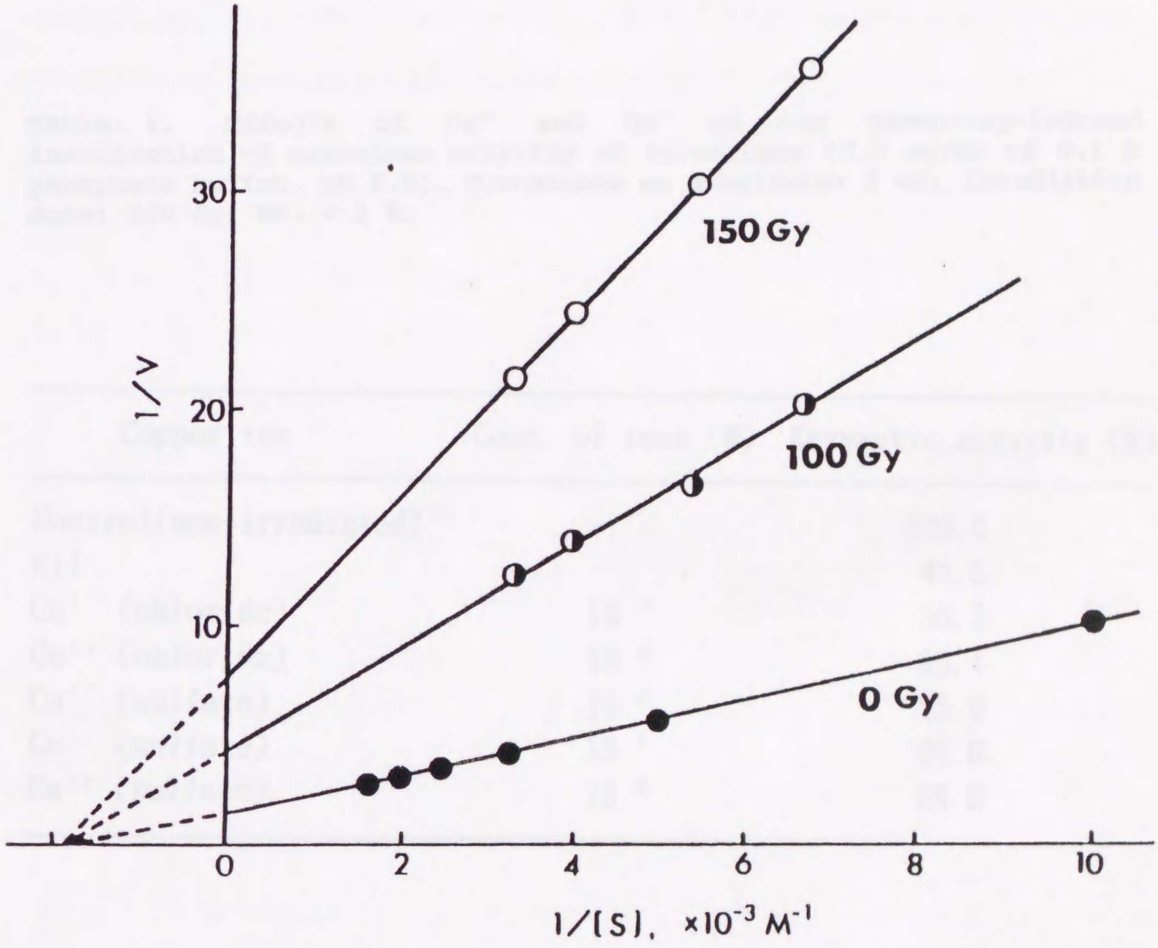

Fig. 2. Lineweaver-Burk plot of cresolase activity of tyrosinase in N_2-saturated solution (0.2 mg/ml of phosphate buffer, pH 6.5) irradiated with ^{60}Co gamma-rays.

Table. 1. Effects of Cu^{++} and Cu^{+} on the gamma-ray-induced inactivation of cresolase activity of tyrosinase (0.2 mg/ml of 0.1 M phosphate buffer, pH 6.5). Tyrosinase as substrate: 2 mM; irradiation dose: 200 Gy; SE: < 2 %.

Copper ion	Conc. of ions (M)	Enzymatic activity (%)
Control(non-irradiated)	—	100.0
Nil	—	47.5
Cu^{+} (chloride)	10^{-4}	50.3
Cu^{++} (chloride)	10^{-4}	95.4
Cu^{++} (sulfate)	10^{-4}	95.0
Cu^{++} (sulfate)	10^{-5}	92.0
Cu^{++} (sulfate)	10^{-6}	89.2

Cu^{++} markedly protected against the radiolytic inactivation of cresolase activity (Fig. 1 and Table 1). Cu^{++} gave a high degree of protection even at a concentration of 10^{-6} M, and there was no difference between the sulfate and chloride. It suggests copper ions only affect the inactivation. On the other hand, Cu^{+} conferred little protection against the inactivation.

DISCUSSION

The ionizing radiation-induced inactivation of many enzymes has been studied previously. The main participant in the inactivation in aqueous system was OH radical in all cases presented.[9] However, in the inactivation of cresolase activity of tyrosinase in the present study, t-BuOH and MeOH as the OH radical scavengers did not affect the inactivation, while N_2O as the e^-_{aq} scavenger had a marked protective effect on the inactivation (Fig. 1). Therefore the main participant was found clearly to be e^-_{aq} for the inactivation of cresolase activity of tyrosinase.

Figure. 1 also shows that the hit number is one, and figure. 2 shows that the K_ms does not change after various dose irradiations. Therefore, there must be only one active center as the inactivation target in the enzyme molecule, which is damaged with the reducing ability of e^-_{aq} but not with the oxidizable OH radical.

In living cells, many kinds of enzymes contain metal ions. Metal ions play roles such as the ionic binder to keep molecular conformations and the center of catalytic activity. Mushroom tyrosinase molecule contains copper ions. Mason[1] proposed a configuration of the copper-oxygen complex of tyrosinase and the oxidation mechanism of monophenol as follows:

$$\text{Protein-Cu}^{+}_{2} + O_2 \rightarrow \text{Protein-Cu}_2\text{-}O_2 \qquad \text{(I)}$$

$$\text{Protein-Cu}_2\text{-}O_2 + \text{monophenol} + 2H^+ \rightarrow \text{Protein-Cu}^{++}_{2} + o\text{-diphenol} + H_2O \qquad \text{(II)}$$

$$\text{Protein-Cu}^{++}_{2} + 2e \rightarrow \text{Protein-Cu}^{+}_{2} \qquad \text{(III)}$$

In the present study, a marked protective effect of Cu^{++} and no protective effect of Cu^{+} on the cresolase activity were found (Fig. 1 and Table 1). Therefore, the above formula must be replaced as follows:

$$\text{Protein-}Cu^{++}_2 + O_2 \rightarrow \text{Protein-}Cu^{+}_2\text{-}O_2 \quad \text{(IV)}$$

$$\text{Protein-}Cu^{+}_2\text{-}O_2 + \text{monophenol} + 2H^{+} \rightarrow \text{Protein-}Cu^{++}_2 + o\text{-diphenol} + H_2O \quad \text{(V)}$$

The inactivation mechanism of cresolase activity is

$$\text{Protein-}Cu^{++}_2 + e^{-}_{aq} \rightarrow \text{Protein-}Cu^{++}Cu^{+} \quad \text{(VI)}$$

The protection effect of Cu^{++} must be due to reaction (VII)

$$Cu^{++} + e^{-}_{aq} \rightarrow Cu^{+} \quad \text{(VII)}$$

or the repair must be due to reaction (VIII).

$$\text{Protein-}Cu^{++}Cu^{+} + Cu^{++} \rightarrow \text{Protein-}Cu^{++}_2 + Cu^{+} \quad \text{(VIII)}$$

It is concluded that the inactivation of cresolase activity is the reduction of Cu^{++} as the active center to Cu^{+} by e^{-}_{aq}.

On the other hand, metal ions such as Fe^{++} and Cu^{++} are able to produce OH radicals by Harber-Weiss cycle ($O_2^{-} + M^{n} \rightarrow O_2 + M^{n-1}$, and 2. $M^{n-1} + H_2O_2 \rightarrow M^{n} + OH^{-} + \cdot OH$).[11, 12] Therefore Cu^{++} can be a sensitizer for the radiation-induced inactivation of non-metalloenzymes. However, in this study, Cu^{++} has no effect on enhancement of the inactivation, but plays a role as a protector of enzymatic activity against radiation. This result supports OH radical is not the main species to inactivate the cresolase activity of tyrosinase.

The enhancement effect of O_2 has been observed in the case of OH-induced inactivation of enzymes.[9] The schema of the enhancement is that the protein radical produced by OH radical reacts with O_2 and results a protein-peroxide

radical. Then the protein-peroxide radical reacts with other protein. In this study, little effect of O_2 was observed (Fig. 1). It supports that the inactivation of cresolase activity is induced by e^-_{aq} but not by OH. In addition, O_2 reacts with e^-_{aq} and produces superoxide ($e^-_{aq} + O_2 \rightarrow O_2^-$). In this case, the main active species is O_2^- for inactivation. However, little enhancement of O_2 indicates little participation of O_2^- in the inactivation.

Finally it is concluded that the active center of cresolase activity is damaged with the reducing ability of e^-_{aq} but not with the oxidizable OH radical. Especially the active center as the main target is cupric ion. Mushroom tyrosinase is constituted of two sets of L and H subunits[14], each LH of which contains two cupric ions, but only one ion reduction by e^-_{aq} must be cause the inactivation of cresolase activity.

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CHAPTER 2.

HYDRATED-INDUCED INACTIVATION OF TYROSINASE IN AQUEOUS SOLUTION BY EXPOSURE TO COBALT-60 GAMMA-RAYS.

II. CATECHOLASE ACTIVITY

ABSTRACT

Tyrosinase (0.2 mg/ml) was irradiated with ^{60}Co gamma-rays. The catecholase activity was measured at varying radiation doses under various atmospheric conditions. D_0 was found to be 1.25 kGy and hit number was found to be 2 in N_2-saturated solution. OH radical scavengers, t-BuOH and MeOH, had no effect. O_2 that is an enhancer of OH-induced enzyme inactivation had little effect. However, N_2O as a e^-_{aq} scavenger and Cu^{++} markedly protected against the inactivation indicating that e^-_{aq} was the main species to inactivate the enzymatic activity. By Ultrogel chromatography, it was found that the enzymatic activity was lost when this enzyme dissociated into its subunits. Thus, it was concluded that the radiation-induced inactivation was due to the reduction of Cu^{++} as the active center and the chelater with e^-_{aq} followed by the dissociation.

INTRODUCTION

Tyrosinase (EC 1. 12. 18. 1) is a copper-containing protein that has two enzymatic activities, these are monophenolmonooxygenase activity (cresolase activity) and o-diphenoloxydase activity (catecholase activity). The enzymes are widely distributed in a lot of organisms from microbials, plants and animals, and contributed the important roles in the metabolism of amino acids and producing of melanin.[1-7] Radiation-induced inactivations of the cresolase activity has been studied as previously reported.[8]

In aqueous systems exposed to radiation, two main active species are produced; OH radicals and e^-_{aq} derived from water molecules.[1-7] OH radicals have been reported to be the main active species responsible for the inactivation of most enzymes on irradiation.[9, 10] In our preceding paper, however, the authors have reported that the cresolase activity of tyrosinase was inactivated by e^-_{aq} but not by OH radicals. The present study performed to determine which species acts as the main inactivator of the catecholase activity which is different from the cresolase activity in the same molecule of tyrosinase.

The preceding study showed that cupric ions (Cu^{++}) protected cresolase activity from inactivation[8], contrary to that Cu^{++} has been shown to sensitize most enzymes to exposure to radiation.[11, 12] The authors also studied here whether Cu^{++} acts as a protector or a sensitizer in the case of catecholase activity inactivation. In cresolase

inactivation, Cu^{++} may play roles to both scavenge e^-_{aq} and/or repair reduced Cu^+ in tyrosinase molecule.[8)]

MATERIALS AND METHODS

Materials:

L-α-(3, 4-Dihydroxyphenyl) alanine (dopa) and catechol were purchased from Wako Pure Chemical Industries (Japan). Mushroom tyrosinase and other chemicals used were the same ones described in our proceeding paper.[8] All the chemicals used were of analytical grade. Triply distilled water was used throughout these studies.

Irradiation:

Preparation of enzyme solutions and irradiation were made by the same method described in our preceding paper.[8]

Enzyme Assay:

The catecholase activity was measured using dopa and catechol as substrates according the previously reported method.[13] By the same method, cresolase activity was measured using tyrosine for comparison. The data presented here are averages of not less than three individual determinations.

Gel Chromatography:

Ultrogel AcA 22 (LKB, Sweden) was used for gel chromatography. 2.5 ml of tyrosinase solution (0.2 mg/ml of 0.1 M phosphate buffer, pH 6.5) was applied onto the column (ϕ 0.9 cm x 45 cm) after irradiation under various atmospheric conditions and was eluted with 0.1 M phosphate buffer, pH 6.5). The enzymatic activity of each fraction

(2.5 ml) was assayed using dopa for catecholase activity and using tyrosine for cresolase activity. Ultrogel chromatography was also performed for a solution of tyrosinase mixed with cysteine (10^{-4} M) without gamma-irradiation.

Construction of Amino Acids:

Two ml of tyrosinase solution (0.2 mg/ml) non-irradiated and irradiated with gamma-rays was dried *in vacuo* and re-dissolved in 800 μl of 5.7 *N* HCl in a glass tube. Air in the tube was replaced with N_2 gas and was then suction-sealed using an aspirator. The solution was heated at 110 °C for 20 h, and the hydrolyzed solution was dried *in vacuo*. After dissolving in 100 μl of amino acid analyzing buffer, pH 2.2, the sample was examined with Beckman 121-M Amino Acid Analyzer. Tryptophan could not be measured by this system.

RESULTS

After irradiating tyrosinase solution under N_2, the catecholase activity was measured using dopa as substrate (Fig. 1). The enzymatic activity decreased in an exponential manner after an initial shoulder. D_0 = 1.25 kGy (37 % surviving dose) and n = 2 (hit number) were found. Catechol was also used as another substrate, the survival curve with which was almost the same as that with dopa (data not shown).

No effect of gamma-ray irradiation on Michaelis-Menten constant, K_m, was found for catecholase activity with either dopa or catechol (data not shown) as also observed in the case of cresolase activity[8] indicating no lowering of affinity with the substrate for the irradiated enzyme.

Effect of O_2, *t*-BuOH, MeOH and N_2O on the inactivation of the enzyme induced by irradiation are shown in Fig. 1. Oxygen, which enhances the OH radical-induced effect, showed little enhancement of inactivation of the enzyme. *t*-BuOH and MeOH, which are OH radical scavengers, were also found to have no effect. However, the presence of an e^-_{aq} scavenger, N_2O, markedly inhibited the inactivation. Therefore, the main species involved in the inactivation of this enzymatic activity must be e^-_{aq} and not OH radicals.

As seen in Fig. 1, Cu^{++} also markedly protected the radiolytic inactivation of catecholase activity. At a radiation dose as 1 kGy, the catecholase activity decreased to about 50 % under N_2. In the presence of Cu^{++}, however,

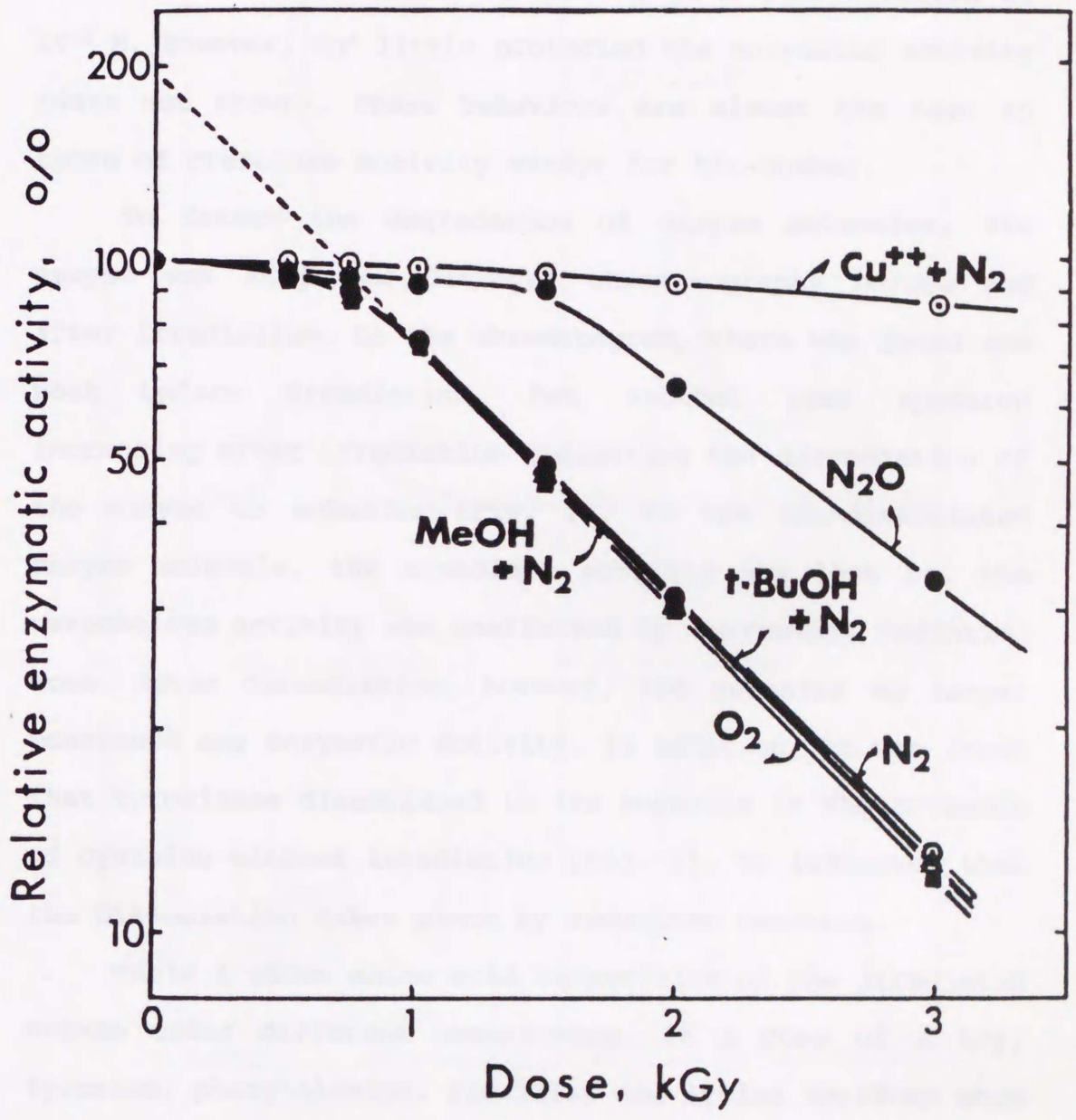

Fig. 1. Survival curve of catecholase activity of tyrosinase in N_2-saturated solution (0.2 mg/ml of phosphate buffer, pH 6.5) irradiated with ^{60}Co gamma-rays, and the effects of the presence of MeOH (10^{-2} M), t-BuOH (10^{-2} M), O_2 (saturated), N_2O (saturated) and Cu^{++} (10^{-4} M). SE: < 2 %.

the activity was maintained at more than 95 % with no difference between the sulfate and chloride at a concentration of 10^{-4} M, and about 90 % even at a concentration of 10^{-6} M. However, Cu^{+} little protected the enzymatic activity (data not shown). These behaviors are almost the same to those of cresolase activity except for hit-number.

To detect the degradation of enzyme molecules, the enzyme was subjected Ultrogel chromatography before and after irradiation. On the chromatogram, there was found one peak before irradiation, but another peak appeared increasing after irradiation indicating the dissociation of the enzyme to subunits (Fig. 2). In the non-dissociated enzyme molecule, the cresolase activity was lost but the catecholase activity was unaffected by increasing radiation dose. After dissociation, however, the subunits no longer possessed any enzymatic activity. In addition, it was found that tyrosinase dissociated to its subunits in the presence of cysteine without irradiation (Fig. 2). It indicates that the dissociation takes place by reducible reaction.

Table 1 shows amino acid composition of the irradiated enzyme under different atmospheres. At a dose of 1 kGy, tyrosine, phenylalanine, histidine and lysine residues were markedly modified especially under N_2O and under $N_2 + Cu^{++}$.

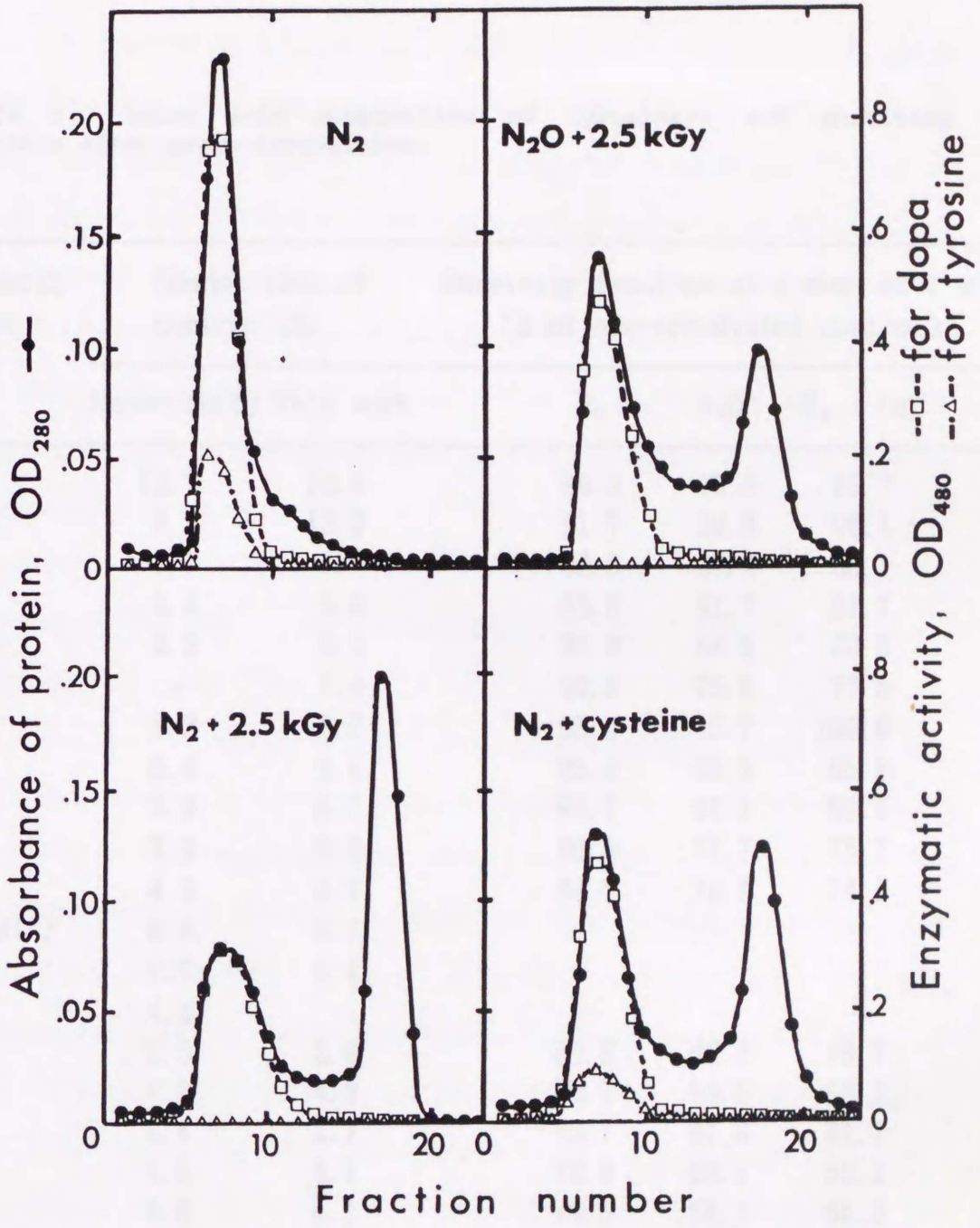

Fig. 2. Dissociation of tyrosinase by irradiation under N_2 or N_2O with gamma-rays compared to that only in the presence if cysteine (10^{-4} M for 30 min). 2.5 ml of tyrosinase solutions (0.2 mg/ml) were eluted with 0.1 M phosphate buffer through an Ultrogel AcA 22 column, are cresolase activity and catecholase activity of each fraction (2.5 ml) were measured using tyrosine and dopa, respectively.

Table. 1. Amino acid composition of tyrosinase and surviving fraction after gamma-irradiation.

Amino acid residue	Composition of control (%)		Surviving fraction at a dose of 1 kGy (% of non-irradiated control)		
	Reported(7)	This work	N_2	N_2O	N_2 + Cu^{++}
Asp	12.7	13.8	99.3	99.9	95.7
Glu	9.5	12.9	91.7	90.8	90.1
Thr	6.1	6.7	92.5	89.2	88.1
Ser	6.4	5.6	96.6	91.7	91.7
Pro	6.2	5.1	93.0	84.9	83.3
ProOH	—	1.4	90.8	75.9	77.5
Gly	8.2	8.2	93.3	95.7	100.0
Ala	6.4	8.4	95.3	96.5	95.8
Val	5.9	6.7	94.1	91.1	92.7
Leu	7.3	6.8	86.0	77.7	75.7
Ile	4.9	4.7	84.7	72.8	74.1
Cys(half)	0.6	0.1	—	—	—
Met	1.7	0.4	—	—	—
Trp	4.2	—	—	—	—
His	2.5	2.0	59.8	42.2	48.7
Phe	4.8	4.3	68.7	53.3	56.1
Tyr	3.4	2.7	72.7	61.2	61.1
Lys	4.6	6.1	72.0	62.5	59.2
Arg	4.6	4.1	78.5	66.7	64.8

(Tyrosinase: 0.2 mg/ml; Cu^{++}: 10^{-4} M; N_2 and N_2O: saturated)

DISCUSSION

It has been found that the ionizing radiation-induced inactivation of cresolase activity of tyrosinase, one of metalloenzyme, is due to e^-_{aq}.[8] This mechanism is different from those of non-metalloenzymes which have been studied as the attack by OH radical derived from H_2O with radiation. In the present study, such e^-_{aq} participation was also found in the inactivation of catecholase activity; that is, the e^-_{aq} scavengers protected the enzymatic activity but the OH radical scavengers did not (Fig. 1). It is therefore concluded that e^-_{aq} is a main active species to inactivate not only the cresolase activity but also the chatecolase activity.

However, there are some differences between the inactivation of catecholase activity and cresolase activity. The catecholase activity (D_0 = 1,250 Gy) of tyrosinase was highly radio-resistant compared to the cresolase activity (D_0 = 350 Gy) under the N_2 gas atmospheres (Fig. 1).[8] Furthermore, the survival curve of the catecholase activity showed n = 2, as hit-number; that is, this activity is lost when two targets are damaged, differing from n = 1 for that of cresolase activity. In other words, the catecholase activity is different from the cresolase activity in the inactivation mechanism. The difference should be due to their functional structures in tyrosinase molecule.

The non-dissociated molecule lost cresolase activity after 2.5 kGy gamma-irradiation under N_2O and N_2

atmospheres (Fig. 2). However, the catecholase activity remained even at considerably higher doses in the non-dissociated molecule. In the presence of cysteine, tyrosinase molecule dissociated to subunits and no longer had the catecholase activity (Fig. 2). This finding indicates that the loss of catecholase activity is due to the dissociation to the subunits differing from the case of inactivation of cresolase activity.

Mason[1] reported the oxidation mechanism of diphenol as follows:

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

Reaction (I) must be modified as follows:

$$\text{subunit-(Cu}^{++}_2\text{)-subunit} + o\text{-diphenol} \rightarrow 2\ \text{subunit-Cu}^{+} + o\text{-quinone} + 2H^{+} \quad \text{(II)}$$

Therefore, the inactivation mechanism of tyrosinase can be as follows:

$$\text{subunit-(Cu}^{++}_2\text{)-subunit} + e^{-}_{aq} \rightarrow \text{subunit-(Cu}^{++}\text{Cu}^{+}\text{)-subunit} \quad \text{(III)}$$

This step is the cresolase activity loss. It is supported by that n = 1 shown in cresolase inactivation.[8]

$$\text{subunit-(Cu}^{++}\text{Cu}^{+}\text{)-subunit} + e^{-}_{aq} \rightarrow 2\ \text{subunit-Cu}^{+} \quad \text{(IV)}$$

This step represents the loss of catecholase activity. It is supported by that n = 2 shown in catecholase inactivation (Fig. 1). Reactions (III) and (IV) are protected or repaired by Cu^{++}.

$$Cu^{++} + e^{-}_{aq} \rightarrow Cu^{+} \quad \text{(V)}$$

This reaction is the protection mechanism.

$$\text{subunit-}(Cu^{++}Cu^{+})\text{-subunit} + Cu^{++} \rightarrow \text{subunit-}(Cu^{++}{}_2)\text{-subunit} + Cu^{+} \quad \text{(VI)}$$

This reaction is the repair mechanism for the cresolase activity.

$$2\ \text{subunit-}Cu^{+} + Cu^{++} \rightarrow \text{subunit-}(Cu^{++}Cu^{+})\text{-subunit} + Cu^{+} \quad \text{(VII)}$$

This reaction is the repair mechanism of the catecholase activity.

As mentioned before, tyrosinase molecule dissociated to the subunits in the presence of cysteine (Cys-SH) (Fig. 2). The presence of cysteine could bring out the dissociation of the tyrosinase molecule, as follows.

$$\text{subunit-}(Cu^{++}{}_2)\text{-subunit} + 2\ \text{Cys-SH} \rightarrow 2\ \text{subunit-}Cu^{+} + \text{Cys-S-S-Cys} + 2H^{+} \quad \text{(VIII)}$$

The dissociation must be due to reducing ability of cysteine like e^{-}_{aq}. It is concluded that both e^{-}_{aq} and cysteine participate in the dissociation to subunits, resulting of the inactivation of not only catecholase activity but also cresolase activity. If disulfide bond participates in the binding of subunits, there must be OH-induced inactivation and O_2 enhancement effect as the oxidizable reaction. Indeed, the degradation of cysteine in the molecule increased in the presence of N_2O (Table 1). However, N_2O has little effect on the inactivation (Fig. 1). Therefore, Cu^{++} must be not only the active center but also the binder between the subunits to keep the active form of tyrosinase molecule. The first reduction of Cu^{++} has to cause the cresolase inactivation (III), and the followed reduction of another Cu^{++} to cause the

dissociation and consequently to result the catecholase inactivation (IV).

e^-_{aq} is much larger than OH radical structurally. The attack of e^-_{aq} would be difficult in the configuration of subunit-($Cu^{++}Cu^+$)-subunit more than in the configuration of subunit-(Cu^{++}_2)-subunit because radio-resistibility of catecholase activity is higher than that of cresolase activity. This mechanism can be supported by the higher D_0 shown in the survival curve (Fig. 1).

Under the dose higher than 1.5 kGy, N_2O lost the protection effect (Fig. 1). This would be due to the consumption of N_2O, as the saturated O_2 mostly consume at 1 kGy.

Generally, radiation-induced inactivation of enzymes has been explained by the attack of OH radicals derived from water molecules.[9, 10] Those previous studies indicated that the enzyme inactivation was caused by the oxidation of histidine, tryptophan or cysteine residues with OH radicals. Sulfur-containing amino acids and aromatic amino acids are very radiosensitive, because they have much higher reaction rate constant with OH radicals.[10] In the case of OH radical as main active species, the enhancement of inactivation with O_2 or Cu^{++} has been reported.[11, 12] However, in the inactivations of tyrosinase with e^-_{aq}, little effect of O_2 was observed despite the enhanced damage of some radiosensitive amino acid residues. On the other hand, the presence of N_2O or Cu^{++} protected the enzyme from inactivation despite the enhancement of amino acid residue damages (Table 1). We concluded from these findings

that the radiation-induced inactivation of tyrosinase is induced by reducible ability of e^-_{aq} but not by oxidizable ability of OH radicals, and that the active center is not an amino acid residue not only cresolase activity but also for catecholase activity. At least the catecholase activity is preserved so long as both Cu^{++} ions as the active center in the molecule are not reduced to Cu^+, even if the molecular structure is partially broken with the degradations of some radiosensitive amino acid residues in the presence of N_2O or Cu^{++}. It is noting that the degradation of amino acid residues is not critical on the enzymatic inactivations.

Previously, it has been reported that histidine residues are involved in the configuration of Cu^{++} in the active center of tyrosinase molecule, and the residues have an interaction with the cysteine residue located in the active center.[14,15] The present study, however, confirmed no critical relationship of histidine or cysteine residue to the active center.

In a previous study of UV-induced inactivation of tyrosinase, Cu^{++} has been also reported to act a protector.[13] UV is also able to cause to produce H_2O-derived active species.[16] Therefore, the present study suggests that the protection manner of Cu^{++} is the same in exposure to both UV and ionizing radiations.

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GENERAL DISCUSSION

The radiation-induced inactivation of tyrosinase, which is one of metalloenzymes was studied for two main purposes. 1. There was no report of the radiation-induced inactivation of metalloenzymes. 2. Tyrosinase is an interesting enzymes which has two kinds of activities, cresolase activity and catecholase activity.

Cresolase activity

(1) MeOH and *t*-BuOH as OH radical scavengers did not protect the inactivation. Oxygen as the oxidation enhancer had little effect. However, N_2O and Cu^{++} as the e^-_{aq} scavengers protected efficiently.

(2) The survival curves of the enzymatic activity under various conditions showed n = 1 as the hit number.

(3) Lineweaver-Burk plot of the enzymatic activity showed that K_m had not varied at different irradiation doses.

(4) There was no difference between $CuSO_4$ and $CuCl_2$ used for the source of Cu^{++} in the protection efficiency. CuCl used for the source of Cu^+ had no protection ability.

The result of (1) indicates that the main active species for the inactivation is e^-_{aq}. The result of (2) indicates that the inactivation was one-hit event. The result of (3) indicates no lowering of affinity with the substrate for the irradiated enzyme. The result of (4) shows that Cu^{++} is protective but Cu^+ is not. Therefore, the enzymatic inactivation must be due to the reducing ability

of e^-_{aq}. The protection reaction must be the elimination of e^-_{aq} with N_2O and Cu^{++}; the reactions are $N_2O + e^-_{aq} \rightarrow N_2 + OH + OH^-$ and $Cu^{++} + e^-_{aq} \rightarrow Cu^+$. It is the most reasonable to conclude that the enzymatic inactivation is due to the reduction of the active center Cu^{++} to Cu^+ with e^-_{aq} in tyrosinase molecule; the reaction is subunit-($Cu^{++}Cu^{++}$)-subunit + e^-_{aq} → subunit-($Cu^{++}Cu^+$)-subunit. Free Cu^{++} must play a role to repair Cu^+ to Cu^{++} in tyrosinase molecule; the reaction is subunit-($Cu^{++}Cu^+$)-subunit + Cu^{++} → subunit-($Cu^{++}Cu^{++}$)-subunit + Cu^+.

Catecholase activity

(1) Effects of all of OH radical scavengers and e^-_{aq} scavengers were the same as those for cresolase inactivation.

(2) Survival curves of the enzymatic activity under various conditions showed n = 2 as the hit number, differing from that of cresolase inactivation.

(3) By gel chromatography after irradiation, the dissociation of tyrosinase molecule to subunits was shown. The dissociation was protected with N_2O. In the presence of cysteine, there was also found the dissociation of tyrosinase molecule without irradiation. The fraction of subunits no longer had any enzymatic activity.

(4) Degradation of amino acids was observed after irradiation. Degradation yield increased in the presence of N_2O or Cu^{++} and O_2 enhancing effect on the degradation was also observed.

(5) Lineweaver-Burk plot of the enzymatic activity showed that K_m had not varied at different irradiation doses.

(6) There was no difference between $CuSO_4$ and $CuCl_2$ used for the source of Cu^{++} in the protection efficiency. CuCl used for the source of Cu^{+} had no protection ability.

The result of (1) indicates that the main active species for the inactivation is also e^-_{aq}. The result of (2) shows that the inactivation is two-hits event; that is, the catecholase activity is resistant more than the cresolase activity against radiation. The results of (3) and (4) indicate that the enzymatic inactivation due to the dissociation of tyrosinase molecule to subunits but not to the degradation of amino acids. The result of (5) indicates no lowering of affinity with the substrate for the irradiated enzyme. The result of (6) shows that Cu^{++} is protective but Cu^{+} is not. Therefore, the enzymatic inactivations was due to the reducing ability of e^-_{aq}. The protection reaction must be the elimination of e^-_{aq} with N_2O and Cu^{++}; the reactions are $N_2O + e^-_{aq} \rightarrow N_2 + OH + OH^-$ and $Cu^{++} + e^-_{aq} \rightarrow Cu^{+}$. It is most reasonable to conclude that the enzymatic inactivation is due to the dissociation of tyrosinase molecule to subunits by the two step reductions with e^-_{aq}; the reactions are subunit-$(Cu^{++}Cu^{++})$-subunit + e^-_{aq} $\rightarrow$ subunit-$(Cu^{++}Cu^{+})$-subunit and subunit-$(Cu^{++}Cu^{+})$-subunit + $e^-_{aq} \rightarrow$ 2 subunit-Cu^{+}. Free Cu^{++} must play a role to repair Cu^{+} to Cu^{++} in tyrosinase molecule; the reaction is 2 subunit-Cu^{+} + $Cu^{++} \rightarrow$ subunit-$(Cu^{++}Cu^{+})$-subunit + Cu^{+}.

In the previous studies, OH radical has been known to main active species for the inactivation of various enzymes. In those studies, the main damaging target for inactivation was radiosensitive amino acid such as cystein, histidine or tryptophan. However, the present study showed that the main damaging target was metal ion(s) in the tyrosinase molecule. Finally, the author concluded that the metalloenzyme which has metal ion(s) as the active center might be inactivated with e^-_{aq} but not with OH radical

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