**Generation Mechanism of Deferoxamine Radical by Tyrosine-Tyrosinase Reaction**

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Nitroxide radical formations of deferoxamine mesylate (DFX) that is used clinically to treat iron-overload patients was examined by a tyrosine-tyrosinase reaction system as models of the H-atom transfer or proton-coupled electron transfer. When DFX was exposed to the tyrosine-tyrosinase reaction, nine-line ESR spectrum (g = 2.0063, hfc; aN = 0.78 mT, aH(2) = 0.63 mT) was detected, indicating that the oxidation of DFX leads to a nitroxide radical. The signal intensity of the DFX radical increased dependently on the concentrations of tyrosine and tyrosinase. The amounts of DMPO-OH spin adducts via the tyrosine-tyrosinase reaction declined with DFX. Furthermore, mass spectra of an extra removed from the tyrosine-tyrosinase reaction mixture showed that the enzyme reactions might not be degradations of DFX. Therefore, there might be two types of DFX reaction passways, which could be through an internal electron transfer from tyrosine and hydrogen absorptions by ·OH directly.

**Keywords** Deferoxamine, ESR, tyrosine, tyrosinase, melanin

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**Introduction**

Deferoxamine mesylate (DFX) is a drug used clinically to treat patients with iron-overload.1,2 Sunlight hypersensitivity is one of several adverse effects associated with DFX.3 However, there are few studies on DFX-induced hypersensitivity of the skin. In general, this hypersensitivity manifests as an inflammatory lesion caused by sunlight, which is a source of ultraviolet (UV) radiation. UV photons which mediate inflammatory damage are related to the generation of reactive oxygen species (ROS) via photosensitization mechanisms.4,6 On the other hand, melanin acts as a photo-protector via its functions as skin pigment7–9 and an antioxidant.10–13 We have studied the melanin formation and scavenging or quenching effect of melanin on superoxide anion (O2•–) and singlet oxygen (1O2)13, as well as radical formation by the tyrosine-tyrosinase reaction that is the initial step in melanin formation.14 Our observations indicate that melanin can serve as a scavenger or a quencher of ROS in keratinocytes, and that the tyrosine-tyrosinase reaction can generate hydroxyl radicals (·OH) and hydrogen atoms (·H). Tyrosinase is an enzyme that catalyzes the biological conversion of tyrosine into dopaquinone with dioxygen at the dinuclear copper active site under physiological conditions.15–17 Tyrosine-tyrosinase has a copper ion at its active center and activates molecular oxygen to generate atomic oxygen, which ultimately induces ·H transfer or proton-coupled electron transfer.15,16 In addition, dicopper-peroxide intermediates formed during the catalytic process of converting tyrosine into dopaquinone may decay to produce ·OH through an internal electron transfer from tyrosine.17 Thus, the tyrosine-tyrosinase reaction is linked to the redox state in which ·H or ·OH functions as a mediator of electron transfers.18 On the other hand, reactions of DFX with peroxynitrite-derived carbonate (CO3•–) and nitrogen dioxide radicals (NO2•) have been reported.19 Alternative mechanisms relate to biochemical and pharmacological actions of DFX via reactions with CO3•– and NO2 radicals.19 However, it is not enough to understand mechanisms of DFX radicals on metabolism linked to H-atom transfer reactions.

In this study, we endeavored to identify DFX radical formation through models of the H-atom transfer or proton-coupled electron transfer such as tyrosine-tyrosinase reaction. Burkitt et al. demonstrated the effects of DFX on ·OH generation during acute iron poisoning by employing electron spin resonance (ESR) spin-trapping.20 Our studies were designed to determine whether DFX reaches sites of melanocyte and/or pigmentations via circulating blood, whereby DFX might be affected by the tyrosine-tyrosinase reaction. In addition, we applied a spectrophotometric method to evaluate the competition between DFX and tyrosine to interact with the tyrosinase complex. Auto-oxidation of dopaquinone results in the production of two intermediate chromophores, dopachrome (Abs.: 475 nm) and indole 5,6-quinone (max. Abs.: 540 nm).21 Furthermore, it provides a method with high sensitivity for analyzing a reaction mixture of the tyrosine-tyrosinase reaction with DFX using a liquid chromatograph/time-of-flight mass spectrometer (TOF-MS). The formation mechanism of the nitroxide radical of...
DFX by the tyrosine-tyrosinase reaction is discussed, in an effort to understand the effects of DFX radicals related to important chemical and biochemical reactions.

**Experimental**

*Test materials and reagents*

Reagents were purchased from the following sources: L-tyrosine, phosphate buffer solution (PB, pH 6.8) from Wako Pure Chemicals (Osaka, Japan); tyrosinase from mushroom, and DFX from Sigma-Aldrich Corp. (St. Louis, MO). All other reagents used were of analytical grade.

**ESR determinations of nitroxide radical generated by tyrosine-tyrosinase reaction with DFX**

Tyrosine was dissolved in 1 M HCl to reach 200 mM. Then 1 mM tyrosine solution was prepared by mixing 5 μL of 200 mM tyrosine solution with 5 μL of 1 M NaOH and 990 μL of PB. DFX was dissolved in ultrapure water to be 100 mM. The reaction mixture was prepared to contain different activity of tyrosinase, 10 μL of 100 mM DFX, different concentrations of tyrosine and PB which was added to adjust to a total volume of 200 μL. Immediately after mixing, the mixture was transferred to an ESR spectrometry cell and the ESR measurement was started after 45 s. The measurement conditions of ESR (JES-FA-100, JEOL, Tokyo, Japan) were as follows: field sweep, 330.80 – 340.80 mT; field modulation frequency, 100 kHz; filed modulation width, 0.07 mT; amplitude, 400; sweep time, 1 min; time constant, 0.1 s; microwave frequency, 9.430 GHz; and microwave power, 4 – 5 mW. DFX radical concentration was calculated based on 5 μM TEMPOL, as a nitroxyl radical standard.

**ESR determinations of ·OH generated by tyrosine-tyrosinase reaction**

The reaction mixture was prepared to contain different volumes of 100 mM DFX, 60 μL of 1 mM tyrosine, 10 μL of 8.9 M DMPO, 4 μL of 100 U/μL tyrosinase and 0.2 M PB which was added to adjust to a total volume of 200 μL. Final concentrations of DFX were 0.5, 1, 2, 3, 5, 10 mM. Immediately after mixing, the mixture was transferred to an ESR spectrometry cell and the ESR measurement was started after 45 s. The measurement conditions of ESR (FR30, JEOL, Tokyo, Japan) were as follows: field sweep, 335.5 – 336.5 mT; field modulation frequency, 100 kHz; filed modulation width, 0.079 mT; amplitude, 400; sweep time, 1 min; time constant, 0.1 s; microwave frequency, 9.40 GHz; and microwave power, 4 mW.

**ESR determinations of ·OH generated from an ultrasound device and the Fenton reaction**

First, a glass tube with the reaction mixture that consisted of 190 μL pure water and 10 μL of 100 mM DFX was set in an original ultrasound device.22 The ultrasound device was operated at 1 MHz for ·OH generation. The reaction mixture was exposed to sonication for 30 s at 25°C. Immediately after the exposure, the mixture was transferred to an ESR spectrometry cell and the ESR measurement was started after 60 s. Secondly, Fenton reaction assay was carried out essentially identical to that described in our previous papers.22 Ninety microliters of 100 mM PB, 50 μL of 2 mM hydrogen peroxide (H2O2), 10 μL of 100 mM DFX, and 50 μL of FeSO4 dissolved in pure water were placed in a test tube and mixed. Each mixture was transferred to an ESR spectrometry cell, and the nitroxide radical of DFX was determined 45 s after the addition of FeSO4. Both measurement conditions for ESR were the same as those in the tyrosine-tyrosinase reaction. In addition, the Fenton...
reaction and ultrasound experiment’s ESR samples showed final concentrations of 5 mM DFX according to the results of the tyrosine–tyrosinase reaction with DFX.

Assays of intermediate chromophores by a spectrophotometric method

Three hundred microliters of 60 μM tyrosine and 300 μL of varying concentrations of DFX or PB were placed in a test tube and mixed. Immediately after the addition of 10 μL of 10 U/μL tyrosinase, each mixture was placed for 5 min at 37°C. Absorbance of dopachrom (475 nm) or indole 5,6-quinone (540 nm) of the mixture was read by UV/Vis recording spectrophotometer (U2010, Hitachi High-Technologies Co., Japan).

Qualitative analysis for DFX degradations by LC-TOF/MS

The reaction mixture was prepared to 10 μL of 100 mM DFX, 60 μL of 1 mM tyrosine, 10 μL of 8.9 M DMPO, 4 μL of 100 U/μL tyrosinase and 0.2 M PB which was same as sample conditions for ESR experiments (a total volume of 200 μL). Ten μL of the reaction mixture was transferred to 990 μL acetonitrile and then filtrated by a 0.20 μm hydrophilic syringe-driven filter unit. We used PerkinElmer UHPLC-TOFMS systems (Flexar FX-15 HPLC, AxION2 TOF, PerkinElmer, Waltham, MA) equipped with PerkinElmer Brownlee Supra column C18 (2.1 μm, 2.1 × 100 mm) at 40 degree in a column oven. With regard to gradient elution, solvent A was 0.1 vol% formic acid-ultrapure water and solvent B was 0.1 vol% formic acid-acetonitrile. Flow rate was 0.3 mL/min, the injection volume was 5 μL, and UV detection was carried out at 230 nm. Electrospray ionization mass spectrometry under a positive ion condition was recorded for 3 min in the m/z region from 100 to 1000 Da with the following instrument parameters: drying gas heater; 300 degrees; nebulizing gas, 80 PSI; and capillary exit voltage, 120 V. In the preliminary experiments, we checked fragmentations of DFX by CID mode under conditions of capillary exit voltage; 170 – 180 V. Our data show mass spectra of fragmentations of DFX (C25H48N6O8, 560.684 g/mol, [M+H]+).
m/z 561), such as m/z 144, 201, 243, 319, 401, 443 (Fig. 1). These fragmentations of DFX are useful for checking degradations of DFX after a radical reaction and the enzyme reactions.

Results and Discussion

We have investigated change in a structure of DFX by a tyrosine-tyrosinase reaction system. Nine-line ESR spectrum (g = 2.0063, hfcc; aN = 0.78 mT, aH(2) = 0.63 mT) was detected in the enzyme reaction mixture (final conc. of tyrosine; 30 μM, final conc. of tyrosinase; 400 U, Fig. 2). The oxidation of DFX leads to a nitroxide radical with the same 9 line.19,20,23 Signal intensity of the DFX radical decreased with time (Fig. 2(a)). In addition, it might be a cycle of DFX and DFX radical because DFX radical was not stable in water. DFX radicals were not detected at DFX and tyrosinase solution without tyrosine (data not shown). Peak-to-peak line widths of DFX radicals of the lowest component and the highest component were 0.21 and 0.41 mT, respectively. The peak-to-peak line width of the highest component was broader than that of the lowest component, because the high molecular weight of tyrosinase (120 – 133 kDa) might have changed the tumbling motion of the DFX radical depending on the rotational correlation time.24 These findings indicated that the DFX radical is one possible radical structure as shown Fig. 2(b).

Figure 3 shows the relation between spin numbers of the DFX radical and concentrations of tyrosine, or those of tyrosinase. Each spin number was calculated by ESR spectrum of the DFX radical 1 min after the enzyme reaction. The spin numbers mean relative concentrations of free radicals. Since conditions of the enzyme reaction mixture were different, the spin number of the DFX radical increased with the concentration of substance and enzyme (Fig. 3). DFX might be oxidized by the enzyme reactions until the end of hydroxylation.

As shown in Fig. 4, the signal intensities of the DFX radical were reduced by DMSO in concentration dependent manner. We have already reported that ·H and ·OH are generated through tyrosine-tyrosinase reaction.14 To examine if ·OH is involved in a downstream of the generation process of DFX radical, ESR signals of DFX radical generated by the tyrosine-tyrosinase reaction were analyzed in the presence of DMSO, a scavenger for ·OH.22 To clarify this point, direct reactions of DFX and ·OH were investigated. We used two simple ways of generating ·OH, one was sonolysis of water by the ultrasonic device and the other was the Fenton reaction. DFX was changed to DFX...
radical by ·OH generated from both the systems (Fig. 5). It is well-known that ·OH is a potent factor on hydrogen abstraction. Thus, DFX radical reaction cannot be related to the one-electron oxidation of DFX. In addition, the amounts of DMPO-OH spin adducts via the tyrosine-tyrosinase reaction declined in a DFX concentration dependent manner (data shown supplementary). These findings suggested that DFX inhibited DMPO and ·OH reaction via tyrosine-tyrosinase reaction.

We examined the spin number of the DFX radical versus concentrations of DFX through tyrosine-tyrosinase reaction (Fig. 6). The spin number of the DFX radical reached the maximum at concentrations of 10 mM DFX, 30 μM tyrosine and 430 U tyrosinase. In a spectrophotometric assay, the amount of dopachrome declined with DFX in a concentration dependent manner. On the other hand, the amount of 5,6-quinone did not differ between conditions with and without DFX (data not shown). Kim et al. discussed if the concentration of DFX is increased to 1 mM or greater, DFX can interfere as ·OH scavenger.23 Furthermore, the amount of DFX radical was inversely related to that of dopachrome under the same scale of DFX concentrations. This was a significant finding because dopachrome loss means the inhibition of tyrosinase by DFX. In other words, DFX might link to tyrosinase instead of tyrosine during the radical formation.

Figure 7 shows extracted ion chromatogram (EIC) and target mass spectra obtained at deferoxamine enzyme reaction mixture 3 min after tyrosine-tyrosinase reaction. The mass spectra measurements were carried out using the PerkinElmer Flexar™ FX-15LC with the PerkinElmer AxiON 2TOF MS with Ultraspray 2™ Dual ESI (Electrospray ionization) with source in positive mode. ESI mass spectrometry under a positive ion condition was recorded for 3 min in the m/z region from 100 to 1000 Da with the following instrument parameters: drying gas heater, 300 degree; nebulizing gas, 80 PSI; and capillary exit voltage, 120 V.

In our conclusion, we found that mechanisms of the DFX radical related to an initial step of melanogenesis. In this study, DFX would be changed in a nitroxyl radical compound via tyrosine-tyrosinase reaction. Since DFX decreased DMPO-OH spin adducts via tyrosine-tyrosinase reaction, the mechanisms on the hydroxylation of tyrosine can be eliminated by DFX as a blocker of tyrosinase.25 In our preliminary tests, the relative ·OH concentrations of tyrosinase reaction, Fenton reaction, and sonication were approximately 1.5, 17, and 13 μM, respectively.25 Thus, the DFX radical can be made from hydrogen abstraction by ·OH directly under high concentrations of ·OH source. In addition, in the case of DFX radical detections under tyrosinase reaction, it is not clear and depends on Lot numbers of tyrosinase vials because amounts of DMPO-OH were lower than those of Fenton reaction and sonication sources. It was suggested that low amounts of ·OH attacked by DFX might be a little effects on DFX radical generation. Therefore, there might be two types of DFX reactions passways, which could be through an internal electron transfer from tyrosine and hydrogen absorptions by ·OH directly. Moreover, melanin can be a target for photoinduced oxygen consumption, or generation of H₂O₂ and ·OH.26,27 If DFX attacks the proton transfer reaction such as tyrosine-tyrosinase reaction, it might be related to important metabolisms of photo sensitizers in organs and blood. In the future, we will need to clarify enhancement of DFX for ray hypersensitivity while melanin and other pigments work as photo sensitizers.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References