Formation of a Hydroxyl Radical from Tar Dye by Photo-Illumination

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When indigo carmine (B-2) was illuminated in the presence of phenylalanine in 0.1 M citrate buffer (pH 4.0), *p*-tyrosine, *m*-tyrosine and *o*-tyrosine were identified as hydroxylated products. However, ten other food colors did not form tyrosine isomers. The hydroxylation of B-2 was pH-dependent, and the maximum rate was found at around pH 4.0. Replacement of air with nitrogen gas completely prevented the formation of tyrosine isomers and the decomposition of B-2. In contrast, oxygen gas accelerated both the hydroxylation and the decomposition.

The addition of superoxide dismutase or catalase to this system prevented hydroxylation. Chemical scavengers of the hydroxyl radical (HO·) prevented the hydroxylation. On the other hand, a singlet oxygen scavenger had no significant effect. The above results suggest that the formatiom of HO· may occur in the photochemical reaction system in the presence of B-2 under aerobic conditions, and that a superoxide radical and hydrogen peroxide may be involved in the HO· formation.

Key words food color; indigo carmine; photochemical reaction; tyrosine isomer; phenylalanine; hydroxyl radical

In Japan, 12 tar dyes are presently permitted as food colors. Food colors are classified as xanthene, azo, triphenylmethane and indigo colors from their chemical structures.

It has been reported that xanthene colors with halogen substituents are responsible for various photochemical reactions.[1] In aqueous solution they act as a photochemical source of singlet oxygen ($^1O_2$), which is formed from the reaction of the color molecule in the excited triplet state with dissolved oxygen.[2] It has been reported that xanthene colors may inhibit several enzymes *in vitro* through photochemical reactions.[3] Similarly, riboflavin produces $^1O_2$ *via* photoactivation of the dye molecule and transfer of the energy to dissolved oxygen.[4] In addition, the generation of a superoxide radical (O_2^-) by photochemical reaction in the riboflavin system has been reported.[5] O_2^- undergoes rapid dismutation to hydrogen peroxide (H_2O_2).[6] O_2^- and H_2O_2 may then interact to form a hydroxyl radical (HO·),[7] one of the strongest biological oxidizing agents, which may also play a significant role in bactericidal activity[8] and the pathology of tissue injury.[9]

HO· generation by biochemical systems has been detected by ESR spectroscopy[10] and gas chromatography,[11] in conjunction with spin-trapping and ethylene production from methionine. Aromatic hydroxylation has also been used to measure HO· levels.[12] The adaption of aromatic hydroxylation assays to detect HO· is simple and convenient, because the hydroxylated compounds thus derived, due to a reaction with HO·, are easily detected by HPLC. We have already reported that the formation of tyrosine isomers from phenylalanine by the photochemical riboflavin system may be caused by HO· formed secondarily from the reaction between O_2^- and H_2O_2, generated in a solution containing riboflavin, by illumination with visible light.[13] However, the formation of HO· by visible light in the presence of food colors has not yet been reported.

In the present study, we examined the HO· generation by photochemical reaction in food colors by using phenylalanine as the HO· trapping agent; the formation of tyrosines was monitored by HPLC.

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Materials and Methods

Reagents Food blue No. 1 (B-1, C.I. 42090, brilliant blue FCF), food blue No. 2 (B-2, C.I. 73015, indigo carmine), food red No. 2 (R-2, C.I. 16185, amaranth), food red No. 3 (R-3, C.I. 45430, erythrosine), food red No. 40 (R-40, C.I. 16035, alurared AC), food red No. 102 (R-102, C.I. 16255, new coccine), food red No. 104 (R-104, C.I. 45410, phloxine), food red No. 105 (R-105, C.I. 45440, rose bengale), food red No. 106 (R-106, C.I. 45100, acid red), food yellow No. 4 (Y-4, C.I. 19140, tartrazine) and food yellow No. 5 (Y-5, C.I. 15985, sunset yellow FCF) used in this study are standard products distributed by the NIHS. L-Phenylalanine, *p*-, *m*- and *o*-tyrosine were obtained from Sigma Chemical Co., superoxide dismutase (SOD) (from bovine erythrocytes, 3800 units/mg), 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) and 1,4-diazabicyclo[2.2.2]octane (Dabco) were from Wako Pure Chemical Industries, and catalase (5000 units/ml) was from Oriental Yeast Co. All other reagents used were of the highest purity commercially available. Highly purified water obtained by a Milli-Q system (Millipore) was used for all analyses.

Photo-Illumination The mixture for the photoreaction of phenylalanine contained 1 mg phenylalanine and 5 mg food colors in 0.1 M citrate buffer at pH 4.0 in a final volume of 2 ml. Samples in a glass cell (10 × 80 mm i.d.) were placed in a glass-walled water bath (Thomas T-105) at 37 °C and illuminated with a 300 W flood lamp (National Ref lamp) at a distance of 15cm from the front surface of the lamp. The lux is 45000 at adistance of 15 cm. After illumination, 100 μl of the mixture was injected into the HPLC.

HPLC Conditions HPLC was conducted on an Inertsil ODS-2 (250 × 4.6 mm i.d.; GL Sciences Inc.) using a Jasco 880-PU intelligent HPLC pump, 802-SC system controller and 851-AS intelligent sampler equipped with a Jasco 870-UV intelligent UV/VIS detector. The eluent was 0.17 M acetic acid containing 0.17 M sodium chloride at a flow rate of 1.0 ml/min. The peak areas of the hydroxylated products detected were calculated using a Sic systeminstrument labchart 180.

Assay of Metal Ions in the Reaction Mixture by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) ICP-AES was performed by Kyoto Koken UOP-1 Mark II. The operating conditions were as follows: RF generator, 1.0 kW at 27.12 MHz; plasma argon flow rate, coolant 13.0 l/min, auxiliary 0.6 l/min, nebulizer 0.39 l/min; slit width, 100 μm × 2 mm, entrance and exit; observation height, 10 mm above the load coil; integration time, 5 s × 3 times; and spectral lines, Fe(II) 238.204 nm, Cu(II) 327.396 nm.

Results and Discussion

Formation of Tyrosines from Phenylalanine by Photo-

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Illumination When a solution of B-2 and phenylalanine in citrate buffer (pH 4.0) was illuminated with visible light, three isomers (*p*-tyrosine, *m*-tyrosine, *o*-tyrosine) were found to be formed. A typical chromatographic pattern of the solution is shown in Fig. 1. No significant hydroxylation occurred on the omission of B-2 from the system or without illumination. However, the other food colors did not give the tyrosine formation.

The rate of photochemical hydroxylation of phenylalanine was dependent on pH, as shown in Fig. 2. The optimum pH of the hydroxylation was found to be around pH 4.0. The formation of tyrosine was determined after the illumination of solutions containing various concentrations of B-2 and phenylalanine (data not shown). The amounts of accumulated tyrosine reached a maximum at 5 mg of B-2 and decreased with increasing concentrations of B-2.

Figure 3 shows the time courses of the formation of *p*-, *m*- and *o*-tyrosines by photo-illumination; the formation increased with illumination time. When *p*-, *m*- or *o*-tyrosine was used as a substrate, no decomposition of tyrosine was observed. These results indicate that no formation of dihydroxyphenylalanine from tyrosines occured.

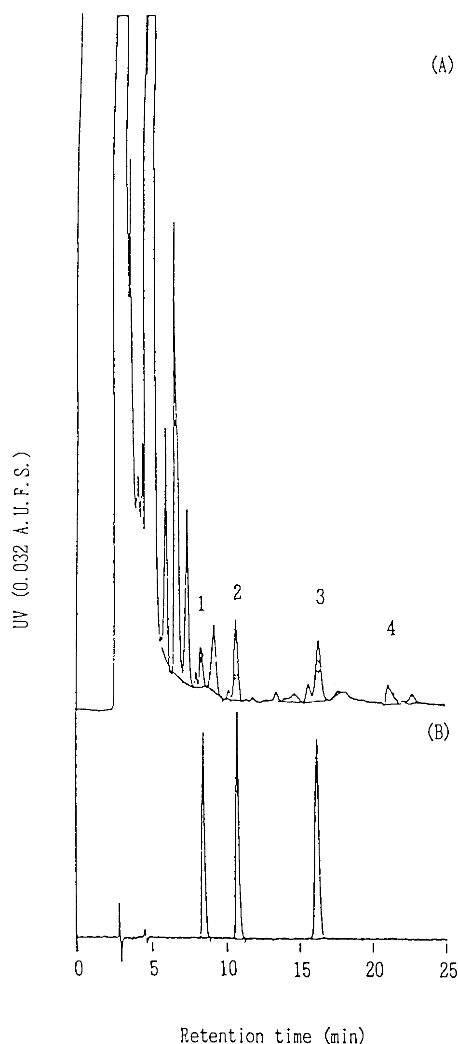

Fig. 1. High Performance Liquid Chromatogram of the Illumination Mixture

(A) After illumination of phenylalanine and B-2 for 90 min, 100 μl of illumination mixture was injected to HPLC. (B) Three μl of solution containing approximately 0.3 μg each of standard compounds was injected to HPLC. HPLC was done with an ultraviolet absorbance monitor at 280 nm. Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

Effectors of the Formation of Tyrosines by Photo-Illumination To investigate the mechanism of the photochemical hydroxylation of phenylalanine in the B-2 system, the following experiments were performed.

Effects of Nitrogen and Oxygen The effects of nitrogen and oxygen on the formation of tyrosines are summarized in Table 1. When nitrogen gas was bubbled through the illumination solution, the formation of tyrosines and the decomposition of B-2 were completely depressed. In contrast, when oxygen gas was bubbled, the formation of tyrosines and the decomposition of B-2 were significantly

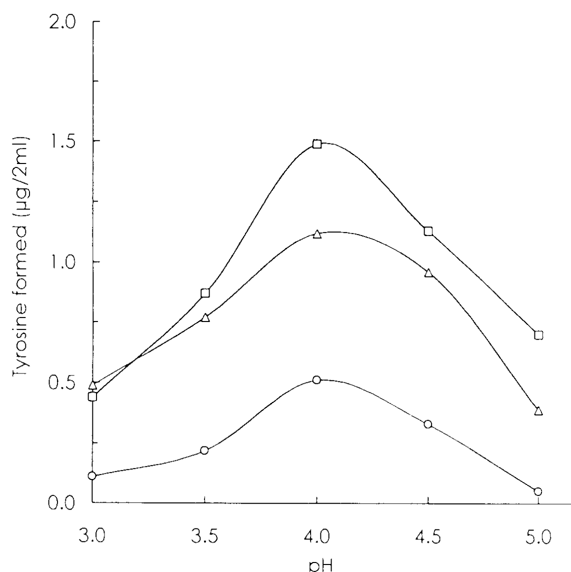

Fig. 2. Effect of pH on the Photochemical Hydroxylation of Phenylalanine in the Presence of B-2

—○—, *p*-tyrosine; —□—, *m*-tyrosine; —△—, *o*-tyrosine. The reaction mixture, containing phenylalanine (1 mg) and B-2 (5 mg) in 0.1 M citrate buffer, in a final volume of 2 ml, was illuminated for 60 min at 37 °C.

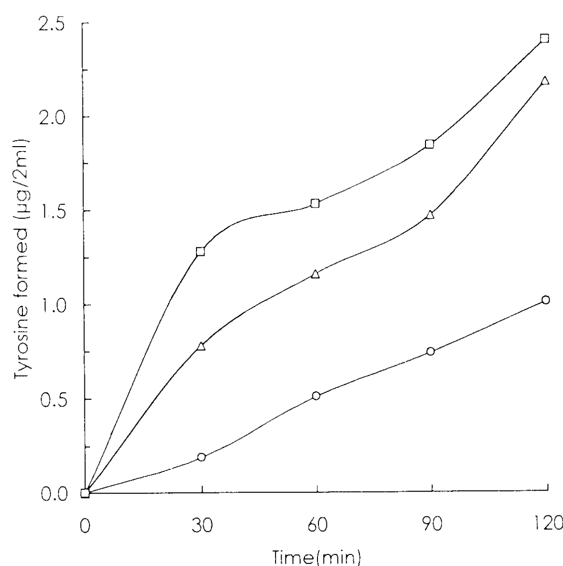

Fig. 3. Time Course of the Photochemical Hydroxylation of Phenylalanine in the Presence of B-2

—○—, *p*-tyrosine; —□—, *m*-tyrosine; —△—, *o*-tyrosine. The reaction mixture, containing phenylalanine (1 mg) and B-2 (5 mg) in 0.1 M citrate buffer, in a final volume of 2 ml, was illuminated at 37 °C.

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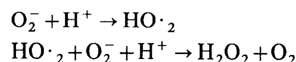
accelerated. These results suggest the participation of molecular oxygen in the formation of tyrosines and the decomposition of B-2.

Effect of Radical Scavengers To obtain evidence that the hydroxylation of phenylalanine by the photochemical reaction system was actually caused by HO·, the effects of some radical scavengers were examined. The result is summarized in Table 2. Hydroxyl radical scavengers such as potassium iodide and potassium bromide effectively prevented tyrosine formation. In contrast, Dabco, employed as a scavenger of $^1O_2$, had no effect on the hydroxylation of phenylalanine. These results suggest that HO· production occurs in the photochemical reaction system under aerobic conditions. The role of O_2^- and H_2O_2 in the HO· formation by the photochemical reaction system was also examined by observing the effect of SOD and catalase on the hydroxylation of phenylalanine. The addition of SOD and catalase also reduced the rate of tyrosine formation. Denatured SOD or catalase, which had been inactivated by boiling, had no effect on the hydroxylation of phenylalanine. Also, a superoxide radical scavenger (Tiron) prevented tyrosine formation.

An optimal hydroxylating reaction was observed at pH 4.0 (Fig. 2). The observed pH profile may reflect the stability of O_2^-.[14] O_2^- is unstable at pH 4—5 at which point H_2O_2 is formed from O_2^- by the following reactions:



$$O_2^- + H^+ \rightarrow HO\cdot_2$$
$$HO\cdot_2 + O_2^- + H^+ \rightarrow H_2O_2 + O_2$$

It has been known that a μM amount of free iron and cupric ions in the medium may be caused in the accelerating effect of hydroxylation.[15] Therefore, we examined the metal ions in the reaction mixture by ICP-AES. Free iron and cupric ions were detected in the quantity of approximately 1 ng/ml and 2 ng/ml, respectively. These results suggest that the metal-catalyzed Harber–Weiss reaction may not have occured.

The results obtained in the present study suggest that HO· production occurs effectively in the photochemical reaction system under aerobic conditions, and O_2^- is the main precursor of HO·. Although the mechanism of HO· formation from O_2^- is obscure, a possible reaction resulting in HO· formation may be the dismutation of O_2^- and the Harber–Weiss[7] reaction, as follows:

$$H_2O_2 + O_2^- \rightarrow HO\cdot + HO^- + O_2$$

Food colors are generally administered to test animals as aqueous solutions in order to study the biological effects of these substances. In the present experiments, the formation of HO· was produced by photo-illumination in the presence of B-2. Since HO· is biologically active, care should be taken to shield the test material from light to prevent the photochemical degradation of the B-2 color.

Table 1. Effects of Nitrogen and Oxygen on the Decomposition of B-2 and the Hydroxylation of Phenylalanine

Condition	Decomposition of B-2 (%)	Rate of tyrosine formation (%)
In air	18	100[a]
In N_2 gas	0	0
In O_2 gas	37	366

The reaction mixture, containing phenylalanine (1 mg) and B-2 (5 mg) in 0.1 M citrate buffer (pH 4.0), was illuminated for 60 min at 37 °C in air, in N_2 gas or in O_2 gas. The decomposition of B-2 was measured and the absorption maximum was at a wavelength of 611 nm. *a*) Tyrosines (3.12 μg/2 ml) formed in air condition (=100%).

Table 2. Effect of Various Substances on the Photochemical Hydroxylation of Phenylalanine in the Presence of B-2

Substance added	Concentration	Rate of tyrosine formation (%)
Complete system	—	100[a]
+Catalase	500 units	3
	100 units	40
+SOD	20 units	48
+Boiled catalase	500 units	100
	100 units	100
+Boiled SOD	20 units	93
+Potassium iodide	1 mM	26
+Potassium bromide	1 mM	64
+Tiron	1 mM	69
+Dabco	1 mM	99

The reaction mixture, containing phenylalanine (1 mg) and B-2 (5 mg) in the presence or absence of the substances listed in 2 ml of 0.1 M citrate buffer (pH 4.0), was illuminated for 60 min at 37°C. Boiled catalase and boiled SOD, prepared by incubation in a boiling water bath for 10 min. *a*) Tyrosines (3.12 μg/2 ml) formed in the absence of the indicated substances (=100%).

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