Formation of a Hydroxyl Radical from Riboflavin Sodium Phosphate by Photo-Illumination

Susumu ISHIMITSU,* Ikuko MISHIMA, Sumiko TSUJI, and Tadashi SHIBATA

National Institute of Health Sciences, Osaka Branch, 1–1–43, Hoenzaka, Chuo-ku, Osaka 540, Japan.
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Photo-illumination of riboflavin sodium phosphate (Rp) with phenylalanine produced significant levels of *o*-tyrosine, *m*-tyrosine and *p*-tyrosine as hydroxylated products. The hydroxylation of Rp was pH-dependent, and the maximum rate was around pH 4.5. Replacement of air with nitrogen prevented the formation of tyrosine isomers while the addition of superoxide dismutase or catalase to this system prevented hydroxylation. The tyrosine formation by the system was significantly prevented by hydroxyl radical (HO·) scavengers such as potassium iodide, potassium bromide, thiourea and sodium formate. No free iron and cupric ions were detected in the reaction mixture by inductively-coupled plasma atomic emission spectrometry. The above results suggest that the formation of HO· may occur in the photochemical reaction system in the presence of Rp under aerobic conditions, and that a superoxide radical and hydrogen peroxide may be involved in HO· formation.

Key words food color; riboflavin sodium phosphate; photochemical reaction; tyrosine isomer; phenylalanine; hydroxyl radical

In Japan, twelve synthetic colors (tar dyes in Japan) and riboflavin (Rf) derivatives are presently permitted as food colors.

As far as food colors are concerned, the formation of active oxygen species such as superoxide radical (O_2^-) and singlet oxygen ($^1O_2$) in visible light has been reported by several authors. Among these active oxygens, O_2^- is known to be generated by visible light in the presence of Rf.[1] The O_2^- undergoes rapid dismutation to hydrogen peroxide (H_2O_2).[2] The Harber–Weiss or transition metal-catalyzed Harber–Weiss reaction of O_2^- and H_2O_2 results in the formation of a hydroxyl radical (HO·),[3] which may also play a significant role in bactericidal activity[4] and the pathology of tissue injury.[5] In addition, the $^1O_2$ is generated by visible light in the presence of xanthene colors with halogen substituents in tar dyes and Rf.[6,7]

The object of our study was to investigate the formation of HO· from food color by photo-illumination. However, the formation of HO· from food color has not previously been demonstrated. As the first step in our study, we reported that the formation of tyrosine isomers from phenylalanine by the photochemical Food Blue No. 2 (B-2, C.I. 73015, indigo carmine) system may be caused by HO· formed secondarily to the reaction between O_2^- and H_2O_2, generated in a solution containing B-2, by illumination with visible light.[8] However, the formation of active oxygen species by visible light in the presence of Rf sodium phosphate (Rp) has not yet been reported. In the present study, we examined HO· production by photochemical reaction of Rp by evaluating the aromatic hydroxylation of phenylalanine as an HO· trapping agent; the formation of tyrosines was monitored by HPLC.

Experimental

Reagents Rp, potassium iodide, potassium bromide, sodium formate, thiourea, 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) and superoxide dismutase (SOD) (from bovine erythrocytes, 3800 units/mg) were obtained from Wako Pure Chemical Industries, *p*-, *m*- and *o*-tyrosine, L-phenylalanine and lumiflavin 3-acetic acid (water-soluble form of lumiflavin) were from Sigma Chemical Co., catalase (5000 units/ml) was from Oriental Yeast Co., and Rf was a standard product distributed by the National Institute of Health Sciences. 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 analysis.

Photo-Illumination The mixture for the photoreaction of phenylalanine contained 3 mM phenylalanine and 0.1 mM Rp in 0.1 M citrate buffer at pH 4.5 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 incandescent lamp at a distance of 15 cm from the front surface of the lamp. The lux is 45000 at a distance of 15 cm. After illumination, 100 μl of the mixture was directly injected into the HPLC.

Detection of HO· HO· has been detected by ESR spectroscopy[9] and gas chromatography,[10] in conjunction with spin-trapping and ethylene production from methional, respectively, but these methods are expensive or complicated. We have demonstrated with chemical systems and HPLC that HO· can mediate the formation of *p*-, *m*- and *o*-tyrosines from phenylalanine, but H_2O_2, O_2^- and $^1O_2$ cannot.[11,12] In this report, we attempted to detect HO·, which may be generated by photochemical reaction of Rp using phenylalanine as the HO· trapping agent and HPLC.

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 free iron and cupric ions in the reaction mixture were measured by ICP-AES under the conditions described previously.[8]

Results and Discussion

Formation of Tyrosines from Phenylalanine by Photo-Illumination of the Rp System A solution of phenylalanine and Rp in citrate buffer (pH 4.5) was illuminated with visible light, and three isomers (*p*-, *m*- and *o*-tyrosine) were found to be formed. A typical chromatogram of the solution is shown in Fig. 1. No significant hydroxylation occurred on the omission of Rp from the system or without illumination. Radical attack on phenylalanine produces three specific products, *p*-, *m*- and *o*-tyrosine. We found that addition of Rp to a solution of phenylalanine resulted in the generation of *p*-, *m*- and *o*-tyrosine in comparable amounts.

The hydroxylation was dependent on pH, as shown in Fig. 2. Optimal hydroxylation was observed at pH 4.5 over the pH range of 4 to 6. This pH-dependence and the formation of tyrosine from Rf and lumiflavin (subsidiary

* To whom correspondence should be addressed.

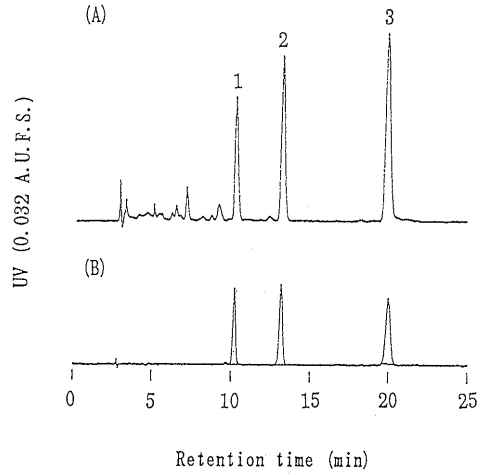

Fig. 1. High Performance Liquid Chromatogram of the Illumination Mixture

(A) After illumination of phenylalanine and Rp for 30 min at 37 °C, 100 μl illumination mixture was injected. (B) Three μl of a solution containing approximately 0.1 μg each of standard compounds was injected. The HPLC detector operated at 280 nm. Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine.

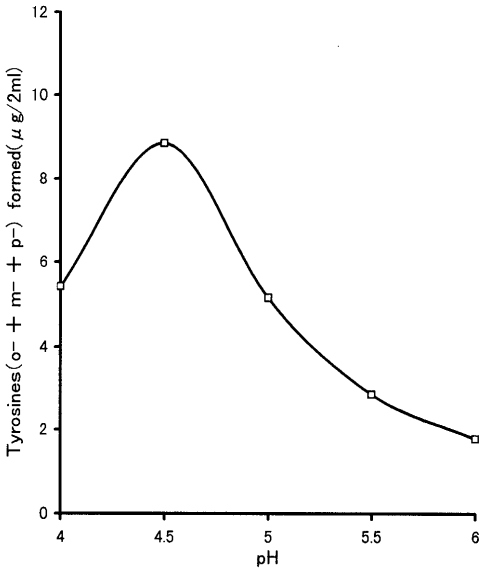

Fig. 2. Effect of pH on the Photochemical Hydroxylation of Phenylalanine in the Presence of Rp

The reaction mixture, containing Rp (0.1 mM) and phenylalanine (3 mM) in 0.1 M citrate buffer, in a final volume of 2 ml, was illuminated for 30 min at 37 °C.

coloring matters of Rf and Rp) were similar to that of the Rp system (data not shown).

Figure 3 shows the time-course of the formation of tyrosines by photo-illumination. No formation of tyrosine was observed until 15 min, and the amount of accumulated tyrosine reached a maximum at 60 min followed by a steady decline over the next 30 min.

Factors Modifying Tyrosine Formation from Phenylalanine by Photo-Illumination of the Rp System To obtain evidence that the hydroxylation of phenylalanine by the photo-illumination of the Rp system was actually caused by HO·, the effects of molecular oxygen and some radical scavengers were examined. Under anaerobic conditions produced by replacement of oxygen with nitrogen, tyrosine formation was approximately 13% that under aerobic conditions. This finding suggests the participation of

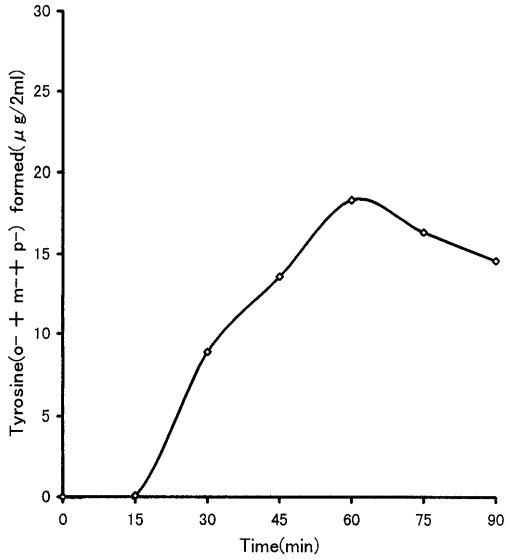

Fig. 3. Time-Course of the Photochemical Hydroxylation of Phenylalanine in the Presence of Rp

The reaction mixture, containing Rp (0.1 mM) and phenylalanine (3 mM) in 0.1 M citrate buffer, in a final volume of 2 ml, was illuminated at 37 °C.

Table 1. Effect of Radical Scavengers on the Photochemical Hydroxylation of Phenylalanine in the Presence of Rp

Substance added	Concentration	Rate of tyrosine formation (%)
Complete system	—	100[a]
+Potassium iodide	1 mM	0
+Potassium bromide	1 mM	11
+Thiourea	1 mM	1
+Sodium formate	1 mM	48
+Catalase	100 units	4
+Tiron	1 mM	35
+SOD	40 units	4

The reaction mixture, containing Rp (0.1 mM) and phenylalanine (3 mM) in the presenc or absence of the substances listed in 2 ml 0.1 M citrate buffer (pH 4.5), was illuminated for 30 min at 37 °C. *a*) Tyrosines (8.86 μg/2 ml) formed in the absence of the indicated substances (= 100%).

molecular oxygen in the hydroxylation of phenylalanine by the photochemical reaction system. The effect of radical scavengers are summarized in Table 1. Hydroxyl radical scavengers such as potassium iodide, potassium bromide, thiourea and sodium formate effectively prevented tyrosine formation. The same results were obtained by the Rf and lumiflavin systems (data not shown). 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 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 strongly inhibited the rate of tyrosine formation, and these inhibitory effects were abolished by heat treatment. An optimal hydroxylating reaction was observed at pH 4.5 (Fig. 2). The observed pH profile may reflect the stability of O_2^-.[13] O_2^- is unstable at pH 4—5, when H_2O_2 is formed from O_2^-. These results suggest the involvement of O_2^- and H_2O_2 in HO· formation by the photochemical reaction system.

To obtain information on the mechanism of HO·

formation by Rp, the inhibitory effects of SOD and catalase on tyrosine formation by the Rp system were compared with those of the Rf and lumiflavin systems. Both SOD and catalase significantly inhibited tyrosine formation by the Rf and lumiflavin systems (data not shown). These compounds have a pteridine ring in their structure. Tetrahydropteridines can easily be oxidized by molecular oxygen, possibly with the formation of H_2O_2.[14] In addition, we have reported the formation of tyrosine isomers from phenylalanine by the autooxidation of 6,7-dimethyl-5,6,7,8-tetrahydropteridine.[15] The results obtained in the present study suggest that HO· production occurs in compounds containing a pteridine ring. Although Food Blue No. 2 has an indoline ring in its structure, the mechanism of the formation of HO· by Food Blue No. 2 has not been clarified. Therefore, further studies are necessary to clarify the mechanism of HO· formation by compounds having an indoline ring.

To determine whether contamination by traces of free metal ions had an effect in the above tyrosine formation, the metal ions in the reaction mixture were examined by ICP-AES. No free iron and cupric ions were detected. Therefore, the so-called transition metal-catalyzed Harber–Weiss reaction may not occur in the photochemical reaction as shown in the following equations.

$$Me^{n+} + H_2O_2 + O_2^- \rightarrow Me^{(n-1)+} + H_2O_2 + O_2$$
$$\rightarrow HO\cdot + Me^{n+} + HO^- + O_2$$

A possible reactions resulting in HO· formation may be dismutation of O_2^- and the Harber–Weiss reaction, as follows,

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
$$H_2O_2 + O_2^- \rightarrow HO\cdot + HO^- + O_2$$

In conclusion, we demonstrated using phenylalanine as an HO· trapping agent that the generation of HO· occurs in food color. In the present experiments, the formation of HO· was produced by photo-illumination in the presence of Rp. It is known that food colors fade in daylight and ultraviolet light,[16,17] and active oxygen species can be detected.[1,6-8] Since HO· is biologically active, care should be taken to protect the test material from light to prevent photochemical degradation of Rf derivatives.

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