

Studies on the Hydroxylation of Phenylalanine by Hydrogen Peroxide in the Presence of Cupric Ions¹⁾

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When phenylalanine was treated with the hydrogen peroxide-cupric ions system in acetate buffer (pH 6.0), *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were identified as hydroxylated products.

Cupric ions markedly accelerated the decomposition of phenylalanine as compared with other transition metal ions.

Radical scavengers, *e.g.*, potassium iodide, dimethyl sulfoxide, formic acid and ethyl alcohol, largely prevented the decomposition of phenylalanine. Superoxide formed from hypoxanthine-xanthine oxidase was found to decompose phenylalanine to some extent. These results suggest that a transient free radical intermediate, formed from hydrogen peroxide, is primarily responsible for the decomposition.

Keywords—hydroxylation of phenylalanine; phenylalanine; hydrogen peroxide-cupric ions system; radical scavenger; hydroxyl radical; superoxide radical; high performance liquid chromatography

In recent years, the formation of activated oxygen species, such as hydroxyl radicals and superoxide, in the autoxidation of ascorbic acid has been reported by many investigators.³⁾ It is also known that activated oxygen formed from ascorbic acid can inactivate enzymes,⁴⁾ viruses⁵⁾ and bacteriophages.⁶⁾ In a previous paper,⁷⁾ we reported that an ascorbic acid-cupric ions system was capable of hydroxylating phenylalanine to give the isomers of hydroxyphenylalanine. We suggested that activated oxygen, formed from hydrogen peroxide generated by the autoxidation of ascorbic acid, is responsible for the hydroxylation of phenylalanine. Our principal purpose here was to elucidate the mechanism of hydroxylation of phenylalanine by activated oxygen.

It has long been postulated that activated oxygen is produced by a hydrogen peroxide-cupric ions system.⁸⁾ The hydroxylation of aromatic compounds, *e.g.*, benzene⁹⁾ and phenyl esters,¹⁰⁾ by hydrogen peroxide-cupric ions has been reported. However, the hydroxylation of phenylalanine has not yet been studied.

The present paper deals with the hydroxylation of phenylalanine by hydrogen peroxide in the presence of cupric ions.

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Experimental

Materials—Special grade hydrogen peroxide and cupric sulfate were obtained from Nakarai Chemicals, Co., Ltd., Kyoto. Xanthine oxidase from cow's milk was purchased from Boehringer Mannheim GmbH, West Germany. Phenylalanine, 3,4-dihydroxyphenylalanine, *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were purchased from Sigma Chemical Co., U.S.A. All other chemicals were of the highest purity available and were used without further purification.

Methods—Phenylalanine and its hydroxylated products were determined by high performance liquid chromatography. The apparatus used was a Hitachi 635-TG high performance liquid chromatograph equipped with an ultraviolet absorbance monitor (260 nm for phenylalanine and 280 nm for 3,4-dihydroxyphenylalanine, *o*-tyrosine, *m*-tyrosine and *p*-tyrosine). A stainless steel column, 2.1×500 mm, packed with Hitachi #3011-C resin, was attached to the apparatus. The column temperature was kept constant at 45° . The mobile phase consisted of equal volumes of 0.025 M sodium acetate and 0.05 M acetic acid. The flow rate was 1 ml per min. Calibration curves were constructed by plotting the ratio of the peak area (half width \times peak height) of each amino acid. A good correlation of peak area was observed in the range of 1–10 nmol of the amino acids. The chromatographic conditions will be reported in detail elsewhere. Determination of ammonia was performed by a modification of the method of Spackman *et al.*,¹¹⁾ using a Hitachi 034 liquid chromatograph.

Reaction with Hydrogen Peroxide—The reaction mixture contained the following components in 25 ml of 0.1 M acetate buffer (pH 6.0): phenylalanine 2×10^{-3} M, cupric ions 1×10^{-3} M and hydrogen peroxide 10^{-1} M. The incubation was carried out at 37° with shaking, with air as the gas phase unless otherwise stated; a 50 μ l aliquot was injected with a microsyringe into the chromatograph, which was equipped with a Hitachi #3011-C column.

Reaction with Hypoxanthine and Xanthine Oxidase¹²⁾—The reaction mixture contained the following components in 25 ml of 0.1 M acetate buffer (pH 6.0): phenylalanine 2×10^{-4} M, hypoxanthine 2×10^{-2} M and xanthine oxidase 0.5 mg.

Results

Decomposition of Phenylalanine by Hydrogen Peroxide and Cupric Ions

When hydrogen peroxide and cupric ions were added to acetate buffer (pH 6.0) containing phenylalanine, decomposition of phenylalanine was observed. No significant decomposition,

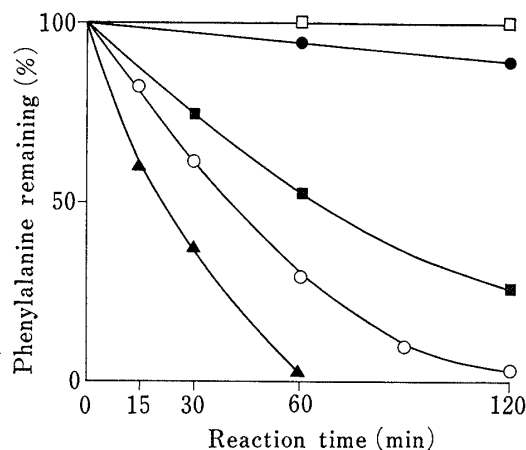


Fig. 1. Decomposition of Phenylalanine in the Presence of Hydrogen Peroxide and Cupric Ions

—□—: phenylalanine + H_2O_2 , phenylalanine + Cu^{2+} (2×10^{-3} M).
 —●—: phenylalanine + H_2O_2 + Cu^{2+} (2×10^{-4} M).
 —■—: phenylalanine + H_2O_2 + Cu^{2+} (5×10^{-4} M).
 —○—: phenylalanine + H_2O_2 + Cu^{2+} (1×10^{-3} M).
 —▲—: phenylalanine + H_2O_2 + Cu^{2+} (2×10^{-3} M).

Phenylalanine (2×10^{-3} M) was incubated with hydrogen peroxide (10^{-1} M) and cupric ions in 0.1 M acetate buffer (pH 6.0) at 37° .

TABLE I. Effects of Metal Ions on the Decomposition of Phenylalanine in the Reaction with Hydrogen Peroxide

Metal ion	Phenylalanine remaining (%)
None	100
Cu^{2+}	29
Fe^{2+}	75
Fe^{3+}	78
Zn^{2+}	98
Ni^{2+}	98
Co^{2+}	100
Mg^{2+}	99
Mo^{2+}	98

Phenylalanine (2×10^{-3} M) was incubated with hydrogen peroxide (10^{-1} M) and metal ions (1×10^{-3} M) in 0.1 M acetate buffer (pH 6.0) for 60 min at 37° .

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12) I. Fridovich, *J. Biol. Chem.*, **245**, 4053 (1970).

however, was observed when one of the two components was omitted from the reaction mixture. The results are shown in Fig. 1.

To examine the effects of other metal ions, transition metal ions were added to the reaction mixture instead of cupric ions and the decomposition of phenylalanine by hydrogen peroxide was determined. The results are summarized in Table I. Only ferrous ions and ferric ions had any effect.

Effectors of the Decomposition of Phenylalanine by Hydrogen Peroxide and Cupric Ions

To investigate the mechanism of the decomposition of phenylalanine by hydrogen peroxide and cupric ions, the following experiments were carried out.

TABLE II. Effects of Radical Scavengers on the Decomposition of Phenylalanine in the Reaction with Hydrogen Peroxide and Cupric Ions

Scavenger	Concentration (M)	Phenylalanine remaining (%)
None	0	29
Potassium iodide	2×10^{-5}	65
Potassium iodide	2×10^{-4}	92
Dimethyl sulfoxide	2×10^{-3}	96
Formic acid	2×10^{-3}	67
Ethyl alcohol	2×10^{-2}	64

Phenylalanine (2×10^{-3} M) was incubated with hydrogen peroxide (10^{-1} M), cupric ions (1×10^{-3} M) and scavengers in 0.1 M acetate buffer (pH 6.0) for 60 min at 37°.

1) **Effect of Radical Scavengers**—Radical scavengers were added to the reaction mixture. As shown in Table II, potassium iodide, dimethyl sulfoxide, formic acid and ethyl alcohol largely prevented the decomposition of phenylalanine by the hydrogen peroxide-cupric ions system. This result indicates that free radicals formed from hydrogen peroxide are responsible for the decomposition of phenylalanine.

2) **Effect of Superoxide on the Decomposition of Phenylalanine**—Superoxide dismutase, which catalyzes the dismutation of superoxide, may play a role as a scavenger of superoxide. Superoxide dismutase isolated from spinach leaves¹³⁾ was therefore added to the hydrogen peroxide-cupric ions system. However, superoxide dismutase was inhibited by hydrogen peroxide. It is known that superoxide is produced by a hypoxanthine-xanthine oxidase system.¹²⁾ We therefore investigated the decomposition of phenylalanine in a hypoxanthine-xanthine oxidase system. The results (Fig. 2) suggest that superoxide formed from the hydrogen peroxide-cupric ions system may also be responsible for the decomposition of phenylalanine.

Products Analysis

To identify the products formed by the reaction of phenylalanine with hydrogen peroxide and cupric ions, the reaction mixture was subjected to liquid chromatography as described earlier. A typical chromatographic pattern is shown in Fig. 3. *o*-Tyrosine, *m*-tyrosine and *p*-tyrosine were formed by the reaction of phenylalanine with hydrogen peroxide and cupric ions. 3,4-Dihydroxyphenylalanine was not detected.

The relationship between the decomposition of phenylalanine and the formation of hydroxyphenylalanine was determined during the course of the reaction. The results obtained are shown in Table III. It was found that phenylalanine is decomposed by hydrogen peroxide in the presence of cupric ions, and that the isomers of hydroxyphenylalanine are produced.

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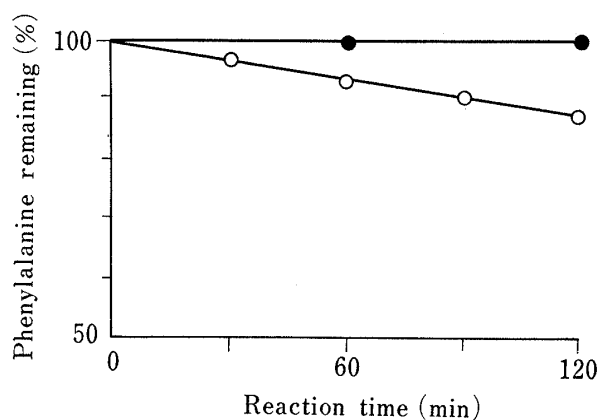


Fig. 2. Decomposition of Phenylalanine by Superoxide

●: phenylalanine + hypoxanthine.
 ○: phenylalanine + hypoxanthine + xanthine oxidase.
 Phenylalanine (2×10^{-4} M) was incubated with hypoxanthine (2×10^{-2} M) and xanthine oxidase (0.5 mg) in 0.1 M acetate buffer (pH 6.0) at 37°.

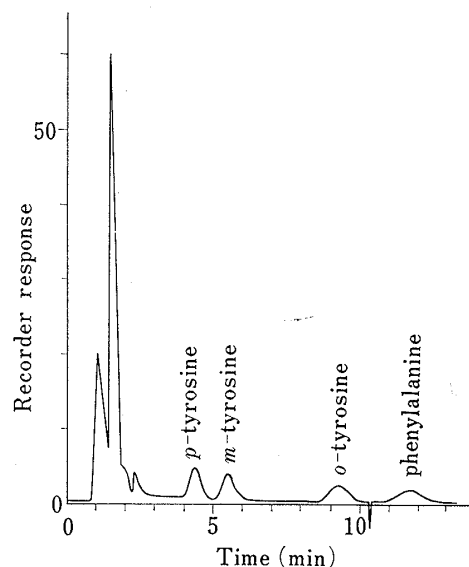


Fig. 3. High Performance Liquid Chromatogram of the Reaction Mixture

After reaction of phenylalanine with hydrogen peroxide and cupric ions for 60 min under the conditions described in "Experimental," 50 μ l of the reaction mixture was subjected to high performance liquid chromatography.

TABLE III. The Hydroxylation of Phenylalanine in the Hydrogen Peroxide-Cupric Ions System

Reaction time (min)	Tyrosine formed (μ mol/25 ml)		
	<i>o</i> -	<i>m</i> -	<i>p</i> -
15	1.1	0.9	0.7
30	2.1	1.5	1.3
60	2.8	2.7	2.0
90	1.0	1.2	1.0
120	0.2	0.4	0.3

The system used for the production of hydroxylated products consisted of cupric ions (25 μ mol), hydrogen peroxide (2500 μ mol) and phenylalanine (50 μ mol) in a volume of 25 ml of 0.1 M acetate buffer (pH 6.0) at 37°.

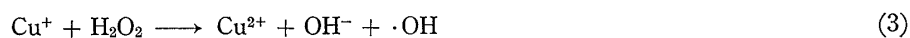
Ammonia was also detected as one of the products. This suggests that deamination also occurred.

Discussion

When phenylalanine was treated with hydrogen peroxide and cupric ions in acetate buffer (pH 6.0), *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were formed.

The hydroxylation was observed in the presence of both hydrogen peroxide and cupric ions, but not in the presence of hydrogen peroxide or cupric ions alone. Among other metal ions tested, only ferrous ions and ferric ions had any effect in place of cupric ions.

The formation of free radicals is known to occur during the reaction of hydrogen peroxide and cupric ions. The mechanism of decomposition of hydrogen peroxide in the presence of cupric ions may be described by the following steps at around pH 6.0⁽⁸⁾;



where $\cdot\text{OOH}$ represents the hydroperoxy radical, $\cdot\text{OH}$ the hydroxyl radical and O_2^- superoxide.

Radical scavengers such as potassium iodide, dimethyl sulfoxide, formic acid and ethyl alcohol prevented the decomposition of phenylalanine. Dixon and Norman¹⁴⁾ reported that the hydroxyl radical has been identified as an intermediate in the hydrogen peroxide-ferrous ions system by electron-spin resonance spectroscopy, whereas the hydroperoxy radical was not detected. Haber and Weiss⁸⁾ reported that the radicals formed from the hydrogen peroxide-cupric ions system and hydrogen peroxide-ferrous ions system were identical. Therefore, it may be concluded that the hydroperoxy radical is not the attacking species.

It is well known that superoxide ions, which are produced in the hydrogen peroxide-cupric ions system, are an active species of molecular oxygen. In this study, the decomposition of phenylalanine was observed by reaction with superoxide ions in the hypoxanthine-xanthine oxidase system (Fig. 2). However, hydroxylated phenylalanine was not detected. These data suggest that superoxide formed in the hydrogen peroxide-cupric ions system is responsible, at least in part, for the decomposition of phenylalanine, but not for its hydroxylation.

On the basis of the above findings, we concluded that the hydroxylation of phenylalanine was caused by hydroxyl radicals produced in the hydrogen peroxide-cupric ions system.

The formation of ammonia was also observed in the hydrogen peroxide-cupric ions system, and further studies on the deamination are in progress.

14) W.T. Dixon and R.O.C. Norman, *Nature*, **196**, 981 (1962).