The Hydroxyl Radical Generated by an Iron(II)/EDTA/Ascorbate System Preferentially Attacks Tryptophan Residues of the Protein

Koji UCHIDA,* Naoki ENOMOTO, Koichi ITAKURA
and Shunro KAWAKISHI

*Department of Food Science & Technology, Nagoya University,
Nagoya 464-01, Japan*

Received July 10, 1989

Iron(II)/EDTA/ascorbate-mediated oxidative damage to specific amino acid residues (tryptophan) of serum albumin was studied. The active species generated by Fe(II)/EDTA/ascorbate preferred to react with tryptophan residues rather than histidine or other amino acids. The observation of preferential damage to tryptophan residues of the protein was fully supported by a model experiment using a tryptophan analogue. The reaction of Fe(II)/EDTA/ascorbate to the protein was significantly suppressed by mannitol and dimethylsulfoxide, suggesting the participation of the hydroxyl radical generated *via* Fenton's reaction. The result was supported by the hydroxyl radical assay using 2-deoxyribose.

Ascorbate is definitely an essential compound present at high concentrations in some mammalian tissues such as adrenals, leucocytes, brain, eyes, and pneumocytes.¹⁾ As a reductant in food and biological systems, ascorbate by itself has been the focus of numerous basic studies.²⁾ However, the beneficial roles of ascorbate have focused attention also on its involvement in detrimental processes, which might be mostly attributed to the autoxidation of ascorbate by itself.³⁾

Ascorbate is relatively stable in pure water, while in the presence of catalytic amounts of metal ion, it is rapidly oxidized to dehydroascorbate through an electron-transfer from ascorbate to metal.^{4~6)} The rate of reaction is known to depend on pH, catalyst, oxygen pressure, temperature, *etc.* The function of ascorbate is to reduce the metal ion $[M(n+1)]$ (Eq. (1)), and to serve as a source for superoxide (O_2^-) (Eq. (2)) and hydrogen peroxide (H_2O_2) (Eq. (3)). The reduced metal ion $[M(n)]$ is conducted via a Fenton's reaction to generate the most potent oxidant, the hydroxyl radical ($\cdot OH$) (Eq. (4)).

Accordingly, the cytotoxicity of ascorbate in the presence of metal ions has been interpreted in terms of the generation of oxygen-derived free radicals.⁷⁾

In vitro, a metal/ascorbate system promotes the oxidative scission of various food and biological materials such as polysaccharides,⁸⁾ proteins,^{7,9~14)} and DNA¹⁵⁾ and also mediates the specific oxygenation of a histamine analogue.¹⁶⁾ Especially, it is of a great interest that this system gives rise to the specific modification of histidine residues of the protein.^{10~14)} We have confirmed that approximately 60% of histidine residues of the protein and peptides were selectively modified

* To whom correspondence should be addressed.

within 24 hr through the reaction with the Cu(II)/ascorbate system.^{13,14)} In relation to this, we have found that active species generated by Fe(II)/EDTA/ascorbate preferentially attack tryptophan residues rather than histidine residues of the protein (K. Uchida, *et al.*, unpublished results).

In this study, we have mainly focused on the reactivity of Fe(II)/EDTA/ascorbate with the tryptophan residues of the protein, compared with Cu(II)/ascorbate. The results indicate that tryptophan residues of the protein and their analogue are particularly susceptible to oxidation by a Fe(II)/EDTA/ascorbate system.

Materials and Methods

Materials. L-Ascorbic acid, ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), dimethylsulfoxide, 2-deoxy-D-ribose, 2-thiobarbituric acid, and mannitol were purchased from Wako Pure Chemical Industries Ltd. (Osaka), and bovine serum albumin from Seikagaku Kogyo Co., Ltd. (Tokyo). *N*-Benzoyl-L-histidine and *N*-*t*-butoxycarbonyl-L-tryptophan were obtained from the Sigma Chemical Company. Trifluoroacetic acid (>99%) was purchased from Tokyo Kasei Kogyo Co., Ltd. All other reagents were of the highest grade commercially available.

Reaction of bovine serum albumin with Fe(II)/EDTA/ascorbate. Reaction mixtures (2.5 ml) containing 0.04% (w/v) bovine serum albumin, 5 mM ascorbate, 50 μM FeSO_4 , and 50 μM EDTA in 0.1 M phosphate buffer (pH 7.2) were incubated at room temperature. After incubation, the reaction was stopped by the addition of 12% trichloroacetic acid (2.5 ml), and the precipitated protein was collected by centrifugation (3000 rpm, 20 min).

The protein was analyzed by HPLC on a TSK-GEL G3000 SW column (7.5 $\times$ 600 mm). Samples were eluted at a rate of 1.0 ml/min with 0.1 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, the elution being monitored continuously at 210 nm.

Slab gels of 10% acrylamide, and Tris-glycine electrophoresis buffer were prepared as described by Davis.¹⁷⁾ Gel sheets were stained with a solution of 0.2% Coomassie Brilliant Blue R-250 in water-2-propanol-acetic acid (5:5:1, v/v/v) and destained with 7% acetic acid containing 10% methanol.

Amino acid analysis of native and oxidized bovine serum albumin was done with a JEOL JLC-6AH amino acid analyzer, for which the samples were prepared as follows: the protein collected by centrifugation was hydrolyzed with 6 N HCl (1.5 ml) at a concentration of 2 mg protein/ml at 120°C for 24 hr. The hydrolyzates were

concentrated, dissolved in 6 ml of aqueous HCl (pH 2.2), and then used for the analysis.

On the other hand, time-dependent change in the tryptophan content of the protein during incubation with Fe(II)/EDTA/ascorbate was measured by the native fluorescence of protein, with a JASCO FP-550A spectrophotometer, by the method of Gutteridge and Wilkins.¹⁸⁾ For this the sample proteins were collected by precipitation with 12% trichloroacetic acid, dissolved with 0.1 M phosphate buffer (5 ml), and their native fluorescence was measured with excitation at 280 nm and emission at 340 nm.

Reaction of the histidine derivative with Fe(II)/EDTA/ascorbate. The histidine derivative, *N*-benzoyl-histidine, was oxidized by incubating 1 mM *N*-benzoyl-histidine in 0.1 M phosphate buffer (pH 7.2, 10 ml) containing 5 mM ascorbate, 50 μM FeSO_4 , and 50 μM EDTA. The samples were incubated at room temperature. *N*-Benzoylhistidine was measured by HPLC on a reversed-phase column. The reaction mixtures were put on a Develosil ODS-5 column (4.6 $\times$ 250 mm) and eluted with 25% methanol in 0.1% trifluoroacetic acid at a rate of 0.8 ml/min, being monitored by absorbance at 230 nm. Areas of the chromatographic peaks of each materials were calculated by a Shimadzu Chromatopac Integrator C-R3A.

Reaction of the tryptophan derivative with Fe(II)/EDTA/ascorbate. Reaction mixtures (10 ml) in phosphate buffer (pH 7.2) containing 1 mM *N*-*t*-butoxycarbonyl-L-tryptophan, 5 mM ascorbate, 50 μM FeSO_4 , and 50 μM EDTA were incubated at room temperature. Both substrate and products were measured by HPLC on a reversed-phase column. The reaction mixture was put on a Develosil ODS-5 column (4.6 $\times$ 250 mm) equilibrated in a solution of 30% methanol in 0.05 M ammonium acetate at a flow rate of 0.8 ml/min. Products were detected by absorbance at 210 nm. Areas of the chromatographic peaks of each materials were calculated by a Shimadzu Chromatopac Integrator C-R3A.

Assay of the hydroxyl radical. The hydroxyl radical was assayed by an adaptation of the method of Aruoma *et al.*¹⁹⁾ The reaction contained, in a final volume of 10 ml of 0.1 M phosphate buffer, pH 7.2, 5 mM ascorbate, 50 μM FeSO_4 , 50 μM EDTA, and 1 mM deoxyribose. The reaction mixture was incubated at room temperature. After incubation, 1.0 ml of 2.8% trichloroacetic acid and 1.0 ml of 1% (w/v) 2-thiobarbituric acid in 0.05 M NaOH were added to 2 ml of the reaction mixture, and the samples were boiled for 10 min, then cooled. The absorbance at 532 nm was measured. The malondialdehyde was calculated based upon a molar extinction coefficient of 1.65×10^5 .

Results

Oxidative modification of the protein by Fe(II)/EDTA/ascorbate

Time dependent alteration of bovine serum albumin during incubation with 50 μM Fe(II), 50 μM EDTA, and 5 mM ascorbate was examined by polyacrylamide gel electrophoresis (Fig. 1). There was a general increase in acidity following exposure to Fe(II)/EDTA/ascorbate. Whether this effect resulted from a loss of basic groups, the formation of new acidic groups, or a combination of the two is uncertain, but active species generated by Fe(II)/EDTA/ascorbate gave rise to some chemical modifications of the amino acid residues of the protein.

Hence, we examined the site of damage of the protein, since $\cdot\text{OH}$ generation at a site-

specific location on the protein molecule has been suggested in metal-catalyzed free radical systems.¹⁸⁾ To find which amino acid residues of the protein are involved in the Fe(II)/EDTA/ascorbate-dependent reactions, bovine serum albumin was treated with Fe(II)/EDTA/ascorbate, hydrolyzed with 6 N HCl, and then used for amino acid analysis. Table I shows that the acid hydrolysis and amino acid analysis. Table I shows that the acid hydrolysis and amino acid analysis found no difference in the amino acid content, other than histidine residues (26.4%), of native and modified protein. However, the fluorescence excitation and emission spectra of the protein showed a marked decrease of the tryptophan content (Fig. 2). Cysteine and methionine residues are likely candidates for oxidative modification, but analysis of the acid hydrolysate found no difference in their contents. Con-

Fig. 1. Polyacrylamide Gel Electrophoresis of Bovine Serum Albumin Exposed to Fe(II)/EDTA/Ascorbate.

The reaction mixtures contained 0.04% bovine serum albumin, 5 mM ascorbate, 50 μM Fe(II), and 50 μM EDTA in 0.1 M phosphate buffer (2.5 ml, pH 7.2), and 0.15 ml portions of the mixtures were put on each time (0, 1, 2, 4, 8, and 24 hr). After the addition of 0.05 ml of glycerol containing 0.01% bromphenol blue, the mixtures were used for polyacrylamide gel electrophoresis. Lanes a and g, zero time; lane b, 1 hr; lane c, 2 hr; lane d, 4 hr; lane e, 8 hr; lane f, 24 hr.

Table I. CHANGES IN AMINO ACID COMPOSITIONS OF BOVINE SERUM ALBUMIN DURING INCUBATION WITH Fe(II)/EDTA/ASCORBATE^a

Amino acid	Molar ratio (%) ^b			
	0 hr	4 hr	8 hr	24 hr
Asp	9.6	9.3	9.5	9.5
Thr	5.5	5.4	5.5	5.6
Ser	4.0	3.7	3.9	4.0
Glu	14.3	14.2	14.0	14.6
Pro	8.1	7.9	8.1	7.8
Gly	2.9	3.0	2.8	3.0
Ala	7.6	7.3	7.4	7.4
Cys	2.0	2.4	2.0	2.0
Val	6.7	6.8	6.6	6.6
Met	0.7	0.8	0.7	0.8
Ileu	2.2	2.4	2.2	2.2
Leu	10.5	10.6	10.2	11.0
Tyr	3.4	3.1	3.3	3.2
Phe	4.7	4.5	4.6	4.8
His	3.8	3.4	3.1	2.8
Lys	10.2	10.6	10.0	10.1
Arg	4.1	4.2	4.1	4.1

^a Reactions were done in phosphate buffer (2.5 ml total volume), pH 7.2, containing 0.04% protein, 5 mM ascorbate, 50 μM FeSO₄, and 50 μM EDTA at room temperature

^b Molar ratio (%) was represented by the molar concentration of each amino acid per total amino acid.

Fig. 2. Time-Dependent Changes in the Fluorescent Properties of Bovine Serum Albumin during Incubation with Fe(II)/EDTA/Ascorbate.

The substrate (0.04%) in 0.1 M phosphate buffer (pH 7.2) was exposed to the Fe(II)/EDTA/ascorbate system composed of 5 mM ascorbate, 50 μ M FeSO₄, and 50 μ M EDTA. Incubation: A, 0 hr; B, 4 hr; C, 8 hr; D, 24 hr.

sidering these results, the oxidative modification of the serum albumin induced by Fe(II)/EDTA/ascorbate involves alteration of specific amino acid residues such as histidine and tryptophan.

Effects of the hydroxyl radical scavengers

It is definite that some active free radical species generated by Fe(II)/EDTA/ascorbate causes these structural alterations of the protein. Actually, mannitol and dimethylsulfoxide, well-known $\cdot$ OH scavengers, significantly suppressed the modification of the protein (Table II). Therefore, the evidence was found to implicate $\cdot$ OH-dependent free radical reactions, while, in contrast, the $\cdot$ OH scavengers tested (mannitol and dimethylsulfoxide) failed to provide any protection from the oxidative modification of the protein by Cu(II)/ascorbate (Table II). This is a serious difference between the Fe(II)/EDTA/ascorbate and the Cu(II)/ascorbate systems.

The results (Table II) led to the assumption that the mechanism of Fe(II)/EDTA/ascorbate is basically distinct from that of Cu(II)/ascorbate. With respect to the reaction of metal/ascorbate with protein, we have report-

Table II. EFFECTS OF THE HYDROXYL RADICAL SCAVENGERS ON THE OXIDATIVE MODIFICATION OF BOVINE SERUM ALBUMIN BY Fe(II)/EDTA/ASCORBATE AND Cu(II)/ASCORBATE^a

Addition	Concentration ^b	% Inhibition ^c	
		Fe(II)/EDTA	Cu(II)
None	—	0	0
Dimethylsulfoxide	10 mM	42	4
Mannitol	10 mM	59	1

^a The reactions were done at room temperature, the solutions containing 0.04% bovine serum albumin, 5 mM ascorbate, and 50 μ M Fe(II)/EDTA or Cu(II) in phosphate buffer (2.5 ml, pH 7.2). Oxidation of the protein was detected by HPLC on a TSK-GEL G3000 SW column.

^b All concentrations shown are final reaction concentrations.

^c The percentage of inhibition is expressed as the inhibition rate of the decrease of the protein peak height on the chromatogram after 24 hr of incubation.

ed that the Cu(II)-dependent system gave rise to selective damage to histidine residues,^{13,14)} and the data have been explained by the ligand formation of histidine with Cu(II) ion. The imidazole ring acts in ligand formation with Cu(II) which might induce the oxidative reaction due to oxygen radicals generated at a site-specific location on the protein molecule. Therefore, lack of inhibition by $\cdot$ OH scavengers of the Cu(II)/ascorbate system has been explained by this site-specific mechanism. But in the Fe(II)/EDTA/ascorbate system, we speculate that Fe(II) ion chelated EDTA reacts with ascorbate without ligand formation with the target molecule (protein) and, therefore, the site of generation of active species is not so restricted as that on the Cu(II)/ascorbate system. It seems probable that selective damage to tryptophan and histidine residues by Fe(II)/EDTA/ascorbate is due to their reactivity with $\cdot$ OH; however, the details remain to be further investigated.

Reaction of the histidine residue analogue with Fe(II)/EDTA/ascorbate

We have measured the reactivity of Fe(II)/EDTA/ascorbate to the histidine de-

Fig. 3. Reactivity of Fe(II)/EDTA/Ascorbate and Cu(II)/Ascorbate toward the Histidine Residues Analogue.

The reaction mixture in 0.1 M phosphate buffer (10 ml total volume), pH 7.2, containing 1 mM *N*-benzoylhistidine, 5 mM ascorbate, and 50 μM Fe(II)/EDTA or Cu(II) was incubated at room temperature. Chromatographic conditions are as follows: column, Develosil ODS-5 (4.6 × 250 mm); eluate, 0.1% trifluoroacetic acid-methanol (4:1); flow rate, 0.8 ml/min; detection, absorbance at 230 nm. Symbols: ○—○, Fe(II)/EDTA/ascorbate; ●—●, Cu(II)/ascorbate.

ivative (*N*-benzoylhistidine), compared with Cu(II)/ascorbate, and it was much lower (Fig. 3). As for products, the reaction of *N*-benzoylhistidine with Fe(II)/EDTA/ascorbate resulted in the loss of the substrate (49%) and, within 24 hr of incubation, a number of products have been detected in low yields (data not shown). They were found to be identical to *N*-benzoylasparagine, *N*-benzoyl-β-(2-oxo-imidazolonyl)alanine, benzamide, *N*-benzoylaspartylurea, *N*-benzoylaspartic acid, *N*-benzoyl-*N*'-formylasparagine, 5-(2-benzamidovinyl)imidazole, and benzoate isolated by the reaction of *N*-benzoylhistidine with Cu(II)/ascorbate.^{20,21)}

Reaction of the tryptophan residue analogue with Fe(II)/EDTA/ascorbate

When the substrate (*N*-benzoylhistidine) was replaced by *N*-*t*-butoxycarbonyl-L-tryptophan, it became apparent that the tryptophan derivative was more reactive with the Fe(II)/EDTA/ascorbate system than the

Fig. 4. Reactivity of Fe(II)/EDTA/Ascorbate and Cu(II)/Ascorbate toward the Tryptophan Residues Analogue.

The reaction mixture in 0.1 M phosphate buffer (10 ml total volume), pH 7.2, containing 1 mM *N*-*t*-butoxycarbonyl-L-tryptophan, 5 mM ascorbate, 50 μM Fe(II)/EDTA or Cu(II) was incubated at room temperature. Chromatographic conditions are as follows: column, Develosil ODS-5 (4.6 × 250 mm); eluate, 0.05 M ammonium acetate-methanol (7:3); flow rate, 0.8 ml/min; detection, absorbance at 210 nm. Symbols: ○—○, Fe(II)/EDTA/ascorbate; ●—●, Cu(II)/ascorbate.

Cu(II)/ascorbate system (Fig. 4). Approximately 66% of the substrate was lost after 24 hr and, instead, a number of newly formed products have been detected. However, their individual yields, based on the substrate, were approximately 5~10% at 24 hr of incubation (data not shown). Isolation and structural elucidation of products are under way and will be presented elsewhere.

Similarly to the result in Table II, the reaction of Fe(II)/EDTA/ascorbate toward the tryptophan derivative was significantly suppressed by $\cdot\text{OH}$ scavengers (dimethylsulfoxide and mannitol) (data not shown). Furthermore, the formation of $\cdot\text{OH}$ from Fe(II)/EDTA/ascorbate was measured by the method of Aruoma *et al.*¹⁹⁾ Figure 5 illustrates the formation of $\cdot\text{OH}$ as thiobarbituric acid-reactive products following the incubation of deoxyribose with Fe(II)/EDTA/ascorbate. The extent of free radical formation by Fe(II)/EDTA/ascorbate was markedly reduced by increasing concentrations of the trypt-

Fig. 5. Assay of the Hydroxyl Radical Generated by Fe(II)/EDTA/Ascorbate Using 2-Deoxyribose as the Substrate.

The reaction mixture containing 1 mM 2-deoxyribose, 5 mM ascorbate, 50 μ M FeSO₄, and 50 μ M EDTA in a final volume of 10 ml of 0.1 M phosphate buffer, pH 7.2, was incubated in the presence and absence of the tryptophan derivative (○—○, 0 mM; △—△, 1 mM; ▲—▲, 10 mM).

tophan derivative. This means that the tryptophan residue analogue is susceptible to the reaction of $\cdot$ OH and competitive with another vulnerable substrate (2-deoxyribose).

Discussion

The iron(II)/EDTA/ascorbate system called the Udenfriend system is a well-known hydroxylating reagent. This system consists of ascorbate, Fe(II), a chelating agent (EDTA), and oxygen, which under physiological conditions of temperature and pH hydroxylates organic substances in a manner shown to be closely analogous to *in vivo* hydroxylations.^{22,23} The hydroxyl radical ($\cdot$ OH) has been regarded as the active species in this system.

The hydroxyl radical is an extremely reactive species that oxidizes cellular constituents or added agents via direct addition (e.g., ring-hydroxylation), hydrogen atom abstraction, and electron transfer. It has been found that a great many biologically relevant compounds have second order reaction rate constants of $10^9 \sim 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ which con-

stitute essentially diffusion-limited reactivity.^{24,25} It is generally believed that an indiscriminate attack on membranes, proteins, essential sulfhydryl groups, and other tissue constituents, is a major reason for tissue damage during *x*-irradiation,²⁶ during exposure *in vivo* to $\cdot$ OH-generating cellular toxins,²⁷ or in the presence of $\cdot$ OH-generating biochemical systems *in vivo*.²⁸ Evidence for the existence of $\cdot$ OH in biochemical systems has been based on the identification of products formed when $\cdot$ OH attacks an exogenous compound added in high concentration.

The results obtained in this study clearly reveal that $\cdot$ OH generated by Fe(II)/EDTA/ascorbate is highly reactive and specific to tryptophan residues of the protein. It has been noted that tryptophan is the most vulnerable amino acid to free radical oxidations,²⁹ and the results agree with known rate constants for reaction of tryptophan with $\cdot$ OH.²⁵ Therefore, it seems probable that the highest reactivity of tryptophan residues of the protein is due to their intrinsic reactivity with the active species ($\cdot$ OH).

On the other hand, for the oxidation of tryptophan residues of protein by iron-redox system, Levine¹⁰ and Kim *et al.*³⁰ have measured the change in tryptophan contents of glutamine synthetase, and they have confirmed that the tryptophan residues of modified protein were not altered. These results are seriously different from our results. Although the reason remains unclear, it is probable that the primary, secondary, and tertiary structure of protein can influence the reactivity of tryptophan residues with $\cdot$ OH generated by iron-redox systems.

The oxidation chemistry of tryptophan and its derivatives is interesting because it is recognized that the indole ring in tryptophan residues is susceptible to oxidation. The formation of *N*-formylkynurenine and kynurenine was confirmed on radiolysis,³¹ photo-oxidation,³² and reaction with lipid hydroperoxide.³³ In metal-catalyzed oxidation systems coupled with H₂O₂, the oxidative inactivation of superoxide dismutase was report-

ed,^{34~36)} and the formation of kynurenine-like compounds has been suggested.³⁶⁾ Oxidized products of tryptophan can contribute to the development of yellow and brown cataracts in the human lens, and, moreover, *N*-formylkynurenine and kynurenine are suspected to be promoters of urinary bladder carcinogenesis in mice.³⁷⁾ In addition, these specific reactions of tryptophan might affect the activity of the enzymes which require it for their functions.

These results demonstrate that $\cdot\text{OH}$ generated by the iron-redox system can essentially modify the protein primary structure. Such modification to primary structure underlie the alteration of secondary and tertiary structure of protein. In relation to this, it has been noted that oxidative modification of primary, secondary, and tertiary structure commonly coincides with increased proteolytic susceptibility.³⁸⁾

The occurrence of similar specific reactions to protein is suggested *in vivo* when the free radical reactions are initiated by iron(II) and ascorbate. It is definite that the formation of $\cdot\text{OH}$ gives rise to selective oxidation of tryptophan residues of protein. Such reactions may therefore be physiologically important in connection with oxygen toxicity and protein turnover in mixed-function oxidation systems.

Based on this study, we are now trying to characterize the oxidation product of the tryptophan residues analogue chemically to establish the mechanism of the reaction of $\cdot\text{OH}$ with tryptophan residues of proteins.

References

- 1) S. Lewin, "Biological Activity and Potential," in Vitamin C: Its Molecular Biology and Medical Potential, Academic, London, 1976, pp. 75~103.
- 2) C. J. Bates, "The Function and Metabolism of Vitamin C in Man," in Vitamin C (Ascorbic Acid), ed. by J. N. Counsell and D. H. Hornig, Applied Science, London, 1981, p. 1.
- 3) B. H. J. Bielski, "Chemistry of Ascorbic Acid Radicals," in Ascorbic Acid: Chemistry, Metabolism, and Uses, ed. by P. A. Seib and B. M. Tolbert, American Chemical Society, Washington, D.C., 1982, pp. 81~100.
- 4) M. M. T. Khan and A. E. Martell, *J. Am. Chem. Soc.*, **89**, 4176 (1967).
- 5) M. M. T. Khan and A. E. Martell, *J. Am. Chem. Soc.*, **89**, 7104 (1967).
- 6) Y. Ogata, Y. Kosugi and T. Morimoto, *Tetrahedron*, **24**, 4057 (1968).
- 7) A. Samuni, J. Aronovitch, D. Godinger, M. Chevion and G. Czapski, *Eur. J. Biochem.*, **137**, 119 (1983).
- 8) K. Uchida and S. Kawakishi, *Agric. Biol. Chem.*, **50**, 2579 (1986).
- 9) E. Shinar, T. Navok and M. Chevion, *J. Biol. Chem.*, **258**, 14778 (1983).
- 10) R. L. Levine, *J. Biol. Chem.*, **258**, 11823 (1983).
- 11) R. L. Levine, *Methods Enzymol.*, **107**, 370 (1984).
- 12) G. Marx and M. Chevion, *Biochem. J.*, **236**, 397 (1985).
- 13) K. Uchida and S. Kawakishi, *Agric. Biol. Chem.*, **52**, 1529 (1988).
- 14) K. Uchida and S. Kawakishi, *J. Agric. Food Chem.*, **37**, 897 (1989).
- 15) S. H. Chiou, *J. Biochem.*, **94**, 1259 (1983).
- 16) K. Uchida, M. Mitsui and S. Kawakishi, *Biochim. Biophys. Acta*, **991**, 377 (1989).
- 17) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
- 18) J. M. C. Gutteridge and S. Wilkins, *Biochim. Biophys. Acta*, **759**, 38 (1983).
- 19) O. I. Aruoma, M. Grootveld and B. Halliwell, *J. Inorg. Biochem.*, **29**, 289 (1987).
- 20) K. Uchida and S. Kawakishi, *Biochem. Biophys. Res. Commun.*, **138**, 659 (1986).
- 21) K. Uchida and S. Kawakishi, *Bioorg. Chem.*, **17**, 330 (1989).
- 22) S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).
- 23) B. B. Brodie, J. Axelrod, P. A. Shore and S. Udenfriend, *J. Biol. Chem.*, **208**, 741 (1954).
- 24) N. Anbar and P. Neta, *Int. J. Appl. Radiat. Isot.*, **18**, 493 (1967).
- 25) L. M. Dorfman and G. E. Adams, National Standard Reference Data System, National Bureau of Standards **46**, Washington D.C., 1973.
- 26) B. H. Bielski and J. M. Gebieki, in "Free Radical in Biology," ed. by W. A. Pryor, Vol. 3, Academic Press, New York, 1977, pp. 2~48.
- 27) G. Chen, *Photochem. Photobiol.*, **28**, 669 (1978).
- 28) K. Brawn and I. Fridovich, *Arch. Biochem. Biophys.*, **206**, 414 (1981).
- 29) K. J. A. Davies, M. E. Delsignore and S. W. Lin, *J. Biol. Chem.*, **262**, 9902 (1987).
- 30) K. Kim, S. G. Rhee and E. R. Stadman, *J. Biol. Chem.*, **260**, 15394 (1985).
- 31) G. G. Jayson, G. Scholes and J. Weiss, *Biochem. J.*, **57**, 386 (1954).
- 32) P. Walrant, R. Santus and L. T. Grossweiner, *Photochem. Photobiol.*, **22**, 63 (1975).
- 33) M. K. Krogull and O. Fennema, *J. Agric. Food Chem.*, **35**, 66 (1987).

- 34) E. K. Hodgson and I. Fridovich, *Biochemistry*, **24**, 5294 (1975).
35) E. K. Hodgson and I. Fridovich, *Biochemistry*, **24**, 5299 (1975).
36) F. Yamakura, *Biochem. Biophys. Res. Commun.*, **122**, 635 (1984).
37) M. Matsushima, S. Takao, E. Erturk and G. T. Bryan, *Cancer Res.*, **42**, 3587 (1982).
38) K. J. A. Davies, *J. Biol. Chem.*, **262**, 9895 (1987).
-