Metal-Catalyzed Oxidative Degradation of Collagen

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Oxidative degradation of collagen by metal-catalyzed free radical generating systems (Cu(II)/ascorbate, Fe(II)/ascorbate, Cu(II)/H_2O_2, and Fe(II)/H_2O_2) has been studied. SDS–PAGE revealed that the reaction of collagen with these systems gave rise to depolymerization of protein. The amino acid analysis of the oxidized collagens indicated significant loss of proline, which was accompanied by the formation of hydroxyproline, aspartic acid, and glutamic acid. In addition, among these free radical generating systems, we found that collagen was most sensitive to oxidation by Cu(II)/H_2O_2. This preferential reactivity of Cu(II)/H_2O_2 was also confirmed by using other prolyl polypeptides such as polyproline and poly(Pro-Gly-Pro). We confirmed that the acid hydrolysis of the oxidized prolyl polypeptide generated considerable amounts of γ-aminobutyric acid other than hydroxyproline and glutamic acid.

INTRODUCTION

The hydroxyl radical, a highly reactive oxidant, has been implicated in peroxide-mediated oxidation of a variety of substrates (Walling, 1975). The univalent reduction of H_2O_2 has been postulated to explain the metal (M^+)-dependent decomposition of H_2O_2 (eq 1). Much attention

$$M^{(n+)} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + OH^- \quad (1)$$

has been given to the iron-dependent decomposition of H_2O_2; however, it has been suggested that the rate constant for the reaction of Cu(I) with H_2O_2 is several orders of magnitude greater than that for Fe(II) (Halliwell and Gutteridge, 1985).

Previous studies have suggested that $^{\bullet}OH$ can have a direct effect on destruction of food and biological components such as protein. They have indicated that a wide variety of reactions can occur on protein molecules, and some of these reactions were found to coincide with increased proteolytic susceptibility (Davies, 1987; Davies and Delsignore, 1987; Davies et al., 1987a,b). We also have investigated the oxidative modification of serum albumin with metal-catalyzed free radical systems such as a metal/ascorbate system (Uchida and Kawakishi, 1988; Uchida et al., 1989) and a metal/H_2O_2 system (Uchida and Kawakishi, 1990). We decided to concentrate our efforts on collagen because of its characteristic compositional features. Collagen, which serves to maintain the physical integrity of the different tissues and organs, is one member of a set of homologous polypeptide chains, each of which is characterized by the presence of a Gly-X-Y repeating sequence throughout 90% or more of its entire length (Miller and Gay, 1982).

It has been shown that collagen is degraded when exposed to active oxygen species such as ozone or hydroxyl radicals (Curran et al., 1984); however, the mechanism of oxidative cleavage of collagen by these oxidants is not yet defined. In the present paper, we have investigated the reaction of soluble collagen with metal-catalyzed free radical generating systems such as Cu(II)/ascorbate, Fe(II)/ascorbate, Cu(II)/H_2O_2, and Fe(II)/H_2O_2 under physiological conditions. In the course of this study, we found that collagen and other prolyl polypeptides were sensitive to oxidation by Cu(II)/H_2O_2 to generate mainly γ-aminobutyric acid (GABA), hydroxyproline, aspartic acid, and glutamic acid after acid hydrolysis of the oxidized substrates.

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MATERIALS AND METHODS

Materials. Type IV collagen (from human placenta), polyproline (MW 10^3–10^4), poly(Pro-Gly-Pro) (MW 2×10^3–10^4), catalase (from bovine liver), and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma Chemical Co. L-Ascorbate, ethylenediaminetetraacetic acid (EDTA) disodium salt, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, mannitol, and dimethyl sulfoxide were obtained from Wako Pure Chemical Industries, Ltd. Hydrogen peroxide (31% w/v) was obtained from Mitsubishi Gas Co. All other reagents were of the highest grade commercially available.

Oxidative Degradation of Collagen and Prolyl Peptides. The solution containing 0.5 mg of collagen, polyproline, or poly(Pro-Gly-Pro) was exposed to 5 mM H_2O_2 or 5 mM ascorbate in the presence of 0.05 mM copper(II) ion or 0.05 mM iron(II), in a total volume of 1 mL of 0.1 M sodium phosphate buffer, pH 7.4. Iron(II) was solubilized with the same concentration of EDTA prior to the addition. The reaction mixture was incubated at 37 °C. The reaction was stopped by the addition of 0.1 mM EDTA for the Cu(II)-catalyzed systems and by the addition of DTPA for the Fe(II)-catalyzed systems. After incubation, the reaction mixtures were dialyzed against distilled water for 24 h, freeze-dried, and then submitted to SDS–PAGE, amino acid analysis, and N-terminal sequence analysis.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). The polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Laemmli (1970), using vertical slab gels. Acrylamide concentration was 4.5% in the spacer gel and 6% in the separating gel. The collagen sample was denatured with 1.2 M urea in the presence of 1% 2-mercaptoethanol for 24 h at 37 °C. 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 Composition. The amino acid analysis was performed with a JEOL JLC-300 amino acid analyzer equipped with a JEOL LC30-DK20 data analyzing system, for which the sample was prepared as follows: collagen and polypeptide samples exposed to free radical generating systems for 24 h at 37 °C were hydrolyzed with 6 N HCl at a concentration of 1 mg/mL for 20 h at 110 °C. The hydrolysates were concentrated, dissolved in aqueous HCl (pH 2.2), and then submitted to amino acid analysis.

Amino-Terminal Sequence Analysis. The amino-terminal sequence analysis was performed on an Applied Biosystems Model 477A gas-phase protein sequencer equipped with an Applied Biosystems Model 120A phenylthiohydantoin analyzer for the on-line detection of phenylthiohydantoin derivatives. The dialyzed collagen sample (approximately 1.0 μg of protein) was denatured

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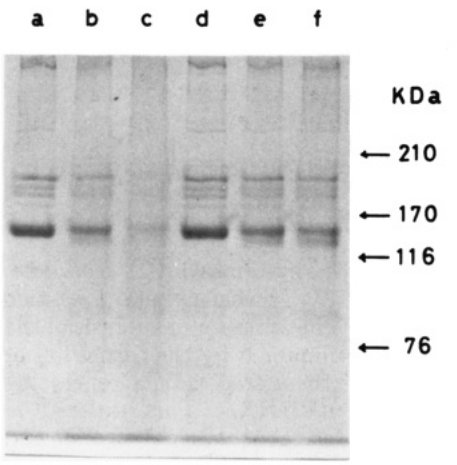

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of collagen exposed to H_2O_2 in the presence of copper(II) or iron(II) ion. The reaction mixture containing collagen (0.5 mg) was incubated with H_2O_2 (5 mM) in the presence of copper(II) ion (0.05 mM) or iron(II) ion (0.05 mM) in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) for 15 or 30 min at 37 °C. Lanes: a and d, control; b, Cu(II)/H_2O_2 (15 min); c, Cu(II)/H_2O_2 (30 min); e, Fe(II)/H_2O_2 (15 min); f, Fe(II)/H_2O_2 (30 min).

with 0.1% SDS for 10 min at 90 °C. Then the sample was loaded onto a trifluoroacetic acid-treated fiber filter. Prior to sample application, the filter was coated with Polybrene and subjected to three cycles of Edman degradation. Anilinothiazolinone derivatives were automatically converted to phenyltiohydantoin derivatives and injected into the on-line analyzer for identification.

RESULTS AND DISCUSSION

First of all, the reaction of soluble collagen with copper- or iron-catalyzed free radical generating systems (Cu(II)/ascorbate, Fe(II)/ascorbate, Cu(II)/H_2O_2, and Fe(II)/H_2O_2) was analyzed by SDS–PAGE. Upon incubation with Cu(II)/H_2O_2 or Fe(II)/H_2O_2, the major bands of the native collagen disappeared within 30 min of incubation (Figure 1). In a similar manner to these results, collagen was sensitive to oxidation by the metal/ascorbate systems (Figure 2). It is evident from these results that Cu(II) is a more effective catalyst for the oxidation of collagen than Fe(II), which coincides with the previous observations by Halliwell and Gutteridge (1985). In addition, we found that collagen underwent depolymerization both in the metal/ascorbate and in the metal/H_2O_2 systems. Although Kano et al. (1987) have reported that the polymerized chains of collagen appeared when collagen was treated with Cu(II)/ascorbate, the polymerization of collagen was not observed in our experiments.

Subsequently, changes in amino acid composition of collagen were assessed to determine which amino acid residues are involved in the oxidative degradation of collagen. As shown in Table I, it is of particular interest to note that Cu(II)/H_2O_2 mediated the most drastic change in amino acid composition of collagen. The result indicates that the loss of proline was the most prominent; approximately 30% was lost in the Cu(II)/H_2O_2 system. The loss of histidine, phenylalanine, lysine, and arginine was also significant. Instead of the decrease of these amino acids, the formation of considerable amounts of hydroxyproline, aspartic acid, and glutamic acid was observed. Poston (1987) has identified *trans*-4-hydroxyproline and *cis*-4-hydroxyproline among the products of poly(L-proline) oxidation. It is definite that hydroxyproline derives from the hydroxylation of proline (Trelstad et al., 1981). However, the source of aspartic acid and glutamic acid

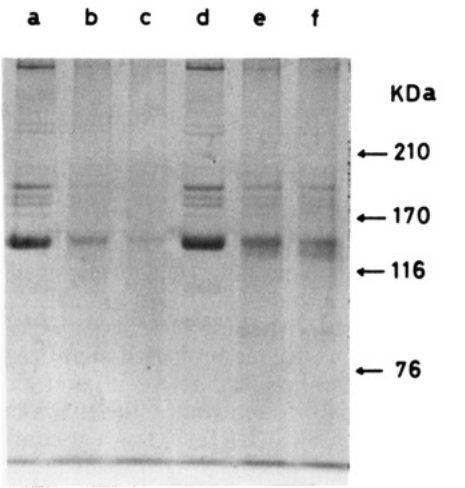

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of collagen exposed to L-ascorbate in the presence of copper(II) or iron(II) ion. The reaction mixture containing collagen (0.5 mg) was incubated with ascorbate (5 mM) in the presence of copper(II) ion (0.05 mM) or iron(II) ion (0.05 mM) in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) for 15 or 30 min at 37 °C. Lanes: a and d, control; b, Cu(II)/ascorbate (15 min); c, Cu(II)/ascorbate (30 min); e, Fe(II)/ascorbate (15 min); f, Fe(II)/ascorbate (30 min).

Table I. Changes in Amino Acid Composition of Collagen[a]

amino acid	molar ratio, %				
	control	Cu/H_2O_2	Fe/H_2O_2	Cu/ASA	Fe/ASA
hypro	11.33	12.64	12.88	11.85	12.14
Asp	5.23	8.21	4.87	6.01	5.35
Thr	2.31	2.13	2.63	2.44	2.15
Ser	3.25	2.95	3.45	3.28	3.05
Glu	8.56	10.09	8.07	8.72	8.27
Pro	8.53	6.35	7.89	7.83	7.76
Gly	31.76	35.63	34.07	33.24	32.98
Ala	5.40	4.83	4.65	5.11	5.30
Cys	0.23	0.43	0.38	0.42	0.49
Val	3.04	2.72	2.91	2.98	3.06
Met	1.18	0.58	0.73	1.05	1.10
Ile	2.57	2.14	2.49	2.58	2.75
Leu	4.53	3.46	4.48	4.44	4.65
Tyr	0.68	0.00	0.68	0.62	0.70
Phe	2.10	0.98	1.71	1.72	1.84
His	0.65	0.21	0.44	0.16	0.55
hylys	4.16	3.29	4.28	3.74	3.94
Lys	1.21	0.56	0.74	0.96	1.15
Arg	3.28	2.60	2.69	2.85	2.79

[a] The reaction mixture containing collagen (0.5 mg) was incubated with ascorbate (5 mM) or H_2O_2 (5 mM) in the presence of copper(II) ion (0.05 mM) or iron(II) ion (0.05 mM) in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 37 °C. Molar ratio (%) was represented by the mole concentration of each amino acid per total amino acid. Hypro, hylys, and ASA represent hydroxyproline, hydroxylysine, and ascorbate, respectively.

has not been established in the oxidation of collagen. But, considering the result in Table I, these amino acids were assumed to arise from proline. Hence, to confirm this prediction, the reaction of prolyl polypeptides with the free radical generating systems was undertaken using polyproline and poly(Pro-Gly-Pro). In a manner similar to collagen, both prolyl peptides were sensitive to oxidation by Cu(II)/H_2O_2 (Tables II and III). Moreover, the acid hydrolysis of the oxidized polypeptides generated considerable amounts of hydroxyproline and glutamic acid. Other than these amino acids, we detected a significant amount of GABA in these experiments. As for the formation of GABA, we have isolated the 2-pyrrolidone compound as the main oxidized product of the proline derivative and demonstrated that the acid hydrolysis of

Table II. Changes in Amino Acid Composition of Polyproline[a]

amino acid	molar ratio, %				
	control	Cu/H_2O_2	Fe/H_2O_2	Cu/AsA	Fe/ASA
hypro	0.00	10.03	9.44	2.11	1.69
Asp	0.01	0.31	0.12	0.06	0.05
Ser	0.01	0.05	0.03	0.00	0.02
Glu	0.00	4.11	1.97	2.32	2.91
Pro	99.38	80.84	85.90	94.13	93.63
GABA	0.01	0.91	0.51	0.55	0.54
ammonia	0.54	2.75	1.64	0.68	0.68

[a] The reaction mixture containing polyproline (0.5 mg) was incubated with ascorbate (5 mM) or H_2O_2 (5 mM) in the presence of copper(II) ion (0.05 mM) or iron(II) ion (0.05 mM) in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 37 °C. Molar ratio (%) was represented by the mole concentration of each amino acid per total amino acid. Hypro, GABA, and ASA represent hydroxyproline, γ-aminobutyric acid, and ascorbate, respectively.

Table III. Changes in Amino Acid Composition of Poly(Pro-Gly-Pro)[a]

amino acid	molar ratio, %				
	control	Cu/H_2O_2	Fe/H_2O_2	Cu/ASA	Fe/ASA
hypro	0.04	4.03	0.38	0.93	0.30
Asp	0.00	0.37	0.10	0.07	0.13
Ser	0.05	0.07	0.04	0.14	0.12
Glu	0.26	4.56	1.07	1.33	2.36
Pro	73.14	57.13	67.77	70.11	68.79
Gly	25.25	25.02	26.46	25.58	25.92
GABA	0.00	1.65	0.29	0.65	0.65
ammonia	0.83	6.53	3.26	1.12	1.53

[a] The reaction mixture containing poly(Pro-Gly-Pro) (0.5 mg) was incubated with ascorbate (5 mM) or H_2O_2 (5 mM) in the presence of copper(II) in (0.05 mM) or iron(II) ion (0.05 mM) in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 37 °C. Molar ratio (%) was represented by the mole concentration of each amino acid per total amino acid. Hypro, GABA, and ASA represent hydroxyproline, γ-aminobutyric acid, and ascorbate, respectively.

the 2-pyrrolidone structure produced GABA quantitatively (Uchida et al., 1990). We therefore consider that GABA detected in Tables II and III derives from the oxidized proline (2-pyrrolidone structure), whereas glutamic acid was identified as the major product in acid hydrolysates of prolyl peptides following their oxidation by •OH (Tables II and III). It is apparent that one of the most probable sources of glutamic acid is proline; however, Dean et al. (1989) have confirmed that proline was not converted into glutamic acid by $Cu(II)/H_2O_2$. Therefore, glutamic acid might be a secondary product produced by the acid hydrolysis of oxidized prolines. In relation to this, Amici et al. (1989) have reported that proline was oxidized with the Fe(II)–EDTA/ascorbate system to generate pyroglutamic acid followed by the formation of glutamic acid with the acid hydrolysis. We also have identified pyroglutamic acid in the oxidation of the proline derivative with $Cu(II)/H_2O_2$ (Uchida et al., 1990). It remained unclear whether glutamic acid is generated only from the oxidation of proline, since a considerable amount of glutamic acid has been detected in the acid hydrolysates of polylysine oxidized with $Cu(II)/H_2O_2$ (data not shown). Moreover, it is apparent from Tables II and III that the yield of aspartic acid was extremely low, suggesting that aspartic acid arose from other amino acids. One source of aspartic acid may be histidine (Dean et al., 1989). We have previously found that the N-protected histidine is converted to aspartic acid through reaction with Cu(II)/ascorbate (Uchida and Kawakishi, 1989) or $Cu(II)/H_2O_2$ (Uchida and Kawakishi, 1990). In addition, Cooper et al. (1985) have also confirmed the formation of a considerable amount of aspartic acid from polyhistidine through reaction with $Cu(II)/H_2O_2$. In the case of collagen, it is extremely doubtful that aspartic acid arises from histidine, since the histidine content in collagen is almost negligible (Table I). Therefore, details about the source of aspartic acid remain to be further investigated.

On the other hand, it is apparent that conversion of proline into other amino acids such as hydroxyproline and pyroglutamic acid does not correlate to the oxidative scission of polypeptide chains by themselves; however, the formation of the 2-pyrrolidone structure results directly in the scission of prolyl peptide chains. Thus, the oxidation of proline residues, which is accompanied by the formation of the 2-pyrrolidone structure, might precede the oxidative cleavage of collagen and other prolyl peptides. In addition, we have found the appearance of a considerable amount of glycine in the N-terminal sequence analysis of native and oxidized collagen by $Cu(II)/H_2O_2$ (data not shown). As glycine is largely predominant in collagen (33% of the residues), this finding does not exclude a random attack of •OH. We suppose that selective formation of glycine as the N-terminal amino acid represents selective damage to the Y residue in the Gly-X-Y repeating sequence of collagen, and the Y residue is assumed to be proline in our experiments.

It is interesting that collagen and prolyl polypeptides are sensitive to oxidation by •OH generated by $Cu(II)/H_2O_2$. We have previously shown that the serum albumin was more sensitive to the oxidation by Cu(II)/ascorbate than that by $Cu(II)/H_2O_2$ (Uchida and Kawakishi, 1988, 1990). Therefore, such reactivity of $Cu(II)/H_2O_2$ might be characteristic for collagen and prolyl polypeptides. At present, we assume that the reactivity of $Cu(II)/H_2O_2$ is due to the affinity of collagen with Cu(II) and H_2O_2. In general, proteins (maybe including collagen) can bind copper ions, which induces the oxidative reaction due to oxygen radicals generated at a site-specific location on the protein molecule. Rowley and Halliwell (1983) have observed that free histidine, or serum albumin, lowered the bulk concentration of •OH in a system in which the radical was generated by a Cu(I)/Cu(II) couple. The Cu(II)–protein complexes might bring about site-specific damage to protein in the presence of H_2O_2, in spite of the tight binding of Cu(II) to the protein molecules. It is possible that H_2O_2 can penetrate into the hydrophobic collagen helix to generate •OH at the Cu(II)-binding site.

In this study, we assessed the oxidation of collagen and proline-containing polypeptides mainly by the amino acid analysis of the hydrolyzed samples. Although our results have the disadvantage of using acid hydrolysis, which modifies some oxidized amino acids, the residues involved and those produced in these reactions of free radicals with protein might be important indicators for assessing the oxidative damage of proteins.

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LITERATURE CITED

Amici, A.; Levine, R. L.; Tsai, L.; Stadtman, E. R. Conversion of Amino Acid Residues in Proteins and Amino Acid Homopolymers to Carbonyl Derivatives by Metal-catalyzed Oxidation Reactions. *J. Biol. Chem.* **1989**, *264*, 3341–3346.

Cooper, B.; Creeth, J. M.; Donald, A. S. R. Studies of the Limited Degradation of Mucus Glycoproteins. *Biochem. J.* **1985**, *228*, 615–626.

Curran, S. F.; Amorso, M. A.; Goldstein, B. D.; Berg, R. A. Degradation of Soluble Collagen by Ozone or Hydroxyl Radicals. *FEBS Lett.* **1984**, *176*, 155–160.

Davies, K. J. A. Protein Damage and Degradation by Oxygen Radicals. I. General Aspects. *J. Biol. Chem.* **1987**, *262*, 9895–9001.

Davies, K. J. A.; Delsignore, M. E. Protein Modification and Degradation by Oxygen Radicals. III. Modification of Secondary and Tertiary Structure. *J. Biol. Chem.* **1987**, *262*, 9908–9913.

Davies, K. J. A.; Delsignore, M. E.; Lin, S. W. Protein Damage and Degradation by Oxygen Radicals. II. Modification of Amino Acids. *J. Biol. Chem.* **1987a**, *262*, 9902–9907.

Davies, K. J. A.; Lin, S. W.; Pacifici, R. E. Protein Damage and Degradation by Oxygen Radicals. IV. Degradation of Denatured Protein. *J. Biol. Chem.* **1987b**, *262*, 9914–9920.

Dean, R. T.; Wolf, S. P.; McElligott, M. A. Histidine and Proline are Important Sites of Free Radical Damage to Proteins. *Free Radical Res. Commun.* **1989**, *7*, 97–103.

Halliwell, B.; Gutteridge, J. M. C. The Importance of Free Radicals and Catalytic Metal Ions in Human Diseases. *Mol. Aspects Med.* **1985**, *8*, 89–193.

Kano, Y.; Sakano, Y.; Fujimoto, D. Cross Linking of Collagen by Ascorbate-Copper Ion Systems. *J. Biochem.* **1987**, *102*, 839–842.

Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Miller, E. J.; Gay, S. Collagen: A Overview. *Methods Enzymol.* **1982**, *82*, 3–32.

Poston, J. M. Effect of Reduced Oxygen Compounds on Poly-L-proline, Proline, and Related Amino Acids. *Fed. Proc.* **1987**, *46*, 1979 (Abstr.).

Rowley, D. A.; Halliwell, B. Superoxide-dependent and Ascorbate-dependent Formation of Hydroxyl Radicals in the Presence of Copper Salts: A Physiologically Significant Reaction? *Arch. Biochem. Biophys.* **1983**, *225*, 279–284.

Trelstad, R. L.; Lawley, K. R.; Holmes, L. B. Nonenzymatic Hydroxylations of Proline and Lysine by Reduced Oxygen Derivatives. *Nature* **1981**, *289*, 310–312.

Uchida, K.; Kawakishi, S. Selective Oxidation of Tryptophan and Histidine Residues in Protein through the Copper-Catalyzed Autoxidation of L-Ascorbic Acid. *Agric. Biol. Chem.* **1988**, *52*, 529–1535.

Uchida, K.; Kawakishi, S. Ascorbate-Mediated Specific Oxidation of the Imidazole Ring in a Histidine Derivative. *Bioorg. Chem.* **1989**, *17*, 330–343.

Uchida, K.; Kawakishi, S. Reaction of a Histidyl Residue Analogue with Hydrogen Peroxide in the Presence of Copper(II) Ion. *J. Agric. Food Chem.* **1990**, *38*, 660–664.

Uchida, K.; Enomoto, N.; Itakura, K.; Kawakishi, S. The Hydroxyl Radical Generated by an Iron(II)/EDTA/Ascorbate System Preferntially Attacks Tryptophan Residues of the Protein. *Agric. Biol. Chem.* **1989**, *53*, 3285–3292.

Uchida, K.; Kato, Y.; Kawakishi, S. A Novel Mechanism for Oxidative Cleavage of Prolyl Peptides Induced by the Hydroxyl Radical. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 265–271.

Walling, C. Fenton's Reagent Revised. *Acc. Chem. Res.* **1975**, *8*, 125–131.

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Registry No. Cu, 7440-50-8; Fe, 7439-89-6; H_2O_2, 7722-84-1; GABA, 56-12-2; Glu, 56-86-0; poly(Pro-Gly-Pro), 25104-46-5; ascorbic acid, 50-81-7; polyproline, 25191-13-3; hydroxyproline, 51-35-4; poly(L-proline) (SRU), 25213-33-6; poly(Pro-Gly-Pro) (SRU), 24937-46-0.