Oxidative Degradation of Collagen and Its Model Peptide by Ultraviolet Irradiation

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The effects of ultraviolet irradiation on collagen and its model peptides were studied. Degradation of collagen was predominant in the system using gel filtration chromatography. The fragmentation was presumably due to oxidation of proline, since collagen is a proline-rich protein and proline residues on collagen markedly decreased with irradiation. To clarify the fragmentation mechanism, poly(L-proline) and $(Pro\text{-}Pro\text{-}Gly)_{10}$ as models of a collagen molecule were used and their oxidation was investigated. Glutamic acid, γ-aminobutyric acid (GABA), and ammonia from the hydrolysates of the irradiated prolyl peptides were identified by amino acid analysis. It was presumed that GABA was generated from a 2-pyrrolidone structure by acid hydrolysis. To confirm this prediction, *N-tert*-butoxycarbonyl (Boc)-L-proline and *N-tert*-Boc-L-prolylglycine were exposed to ultraviolet light, and the irradiation products were isolated and characterized. Then, *N-tert*-Boc-2-pyrrolidone was identified from both UV-irradiated *N-tert*-Boc-L-proline and *N-tert*-Boc-L-prolylglycine. We proposed that the formation of the 2-pyrrolidone compound must contribute to the fragmentation of prolyl peptide on the basis of its structural property.

INTRODUCTION

Ultraviolet light is widely used for sterilization. However, the effect of ultraviolet irradiation on protein components in food was unknown in detail, although that of γ-irradiation on protein was examined by many workers. Previously, photolysis of peptides was investigated using ESR (Lion et al., 1980; Riesz and Rosenthal, 1981; Sevilla and D'Arcy, 1978), flash photolysis (Mittal et al., 1973), and chemical product analysis (Hill et al., 1991). Photooxidation of aliphatic amino acids and peptides was found to lead primarily to photoinduced decarboxylation (Mittal et al., 1973). Photooxidation of aromatic peptides also caused decarboxylation as a result of charge transfer from the aromatic group to the carbonyl group (Sevilla and D'Arcy, 1978).

Collagen is the major component of various tissues and organs. In view of its biological, biochemical, and biomedical importance, many workers have investigated collagen modification with glycation (Sell and Monner, 1989; Brennan, 1989) and oxidation systems. Oxidation of collagen was initiated by ozone (Curran et al., 1984), superoxide anion (Monboisse et al., 1983, 1988), ultraviolet light (Cooper and Davidson, 1965, 1966; Davidson and Cooper, 1967; Miyata et al., 1971; Fujimori, 1965, 1966, 1985, 1988), and hydroxyl radical which was generated with the Fenton reaction (Curran et al., 1984; Kano et al., 1987) or γ-irradiation (Bowes and Moss, 1962). Oxidative fragmentation of the collagen molecule was observed in various oxidation systems (Curran et al., 1984; Monboisse et al., 1983; Bowes and Moss, 1962), while cross-linking was also found (Kano et al., 1987). In early studies, the effect of ultraviolet irradiation on collagen was investigated, and the irradiation caused progressive degradation of collagen into smaller molecular fragments (Cooper and Davidson, 1965, 1966; Davidson and Cooper, 1967; Miyata et al., 1971) or photopolymerization (Fujimori, 1965, 1966, 1985, 1988).

We have examined the oxidative modification of protein and peptide (Uchida and Kawakishi, 1988, 1990; Uchida et al., 1989) and found that collagen was easily degraded by metal-catalyzed oxidation systems (Uchida et al., 1990, 1992). In this work we have investigated the photooxidation of soluble collagen and its model peptides. As a result of irradiation of its model peptide, it seems that oxidative fragmentation of proline-containing peptides was induced by proline oxidation, accompanied by the formation of a 2-pyrrolidone structure.

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MATERIALS AND METHODS

Materials. Collagen (from human placenta), poly(L-proline) (MW 10 000–30 000), horseradish peroxidase, *N-tert*-butoxycarbonyl (Boc)-L-proline, and L-prolylglycine were purchased from Sigma Chemical Co. Superoxide dismutase (from beef erythrocytes) was purchased from ICN Biomedicals, Inc. Deuterium oxide, *tert*-butyl *S*-(4,6-dimethylpyrimidin-2-yl)thiocarbonate, and 3,4-dehydro-L-proline were obtained from Aldrich. $(Pro\text{-}Pro\text{-}Gly)_{10}\cdot 9H_2O$ was purchased from Peptide Institute, Inc. 2-Pyrrolidone and di-*tert*-butyl dicarbonate were obtained from Wako Pure Chemical Industries Ltd. 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) was purchased from Nacarai Tesque, Inc. All other reagents were of the highest grade commercially available.

Irradiation of Collagen and Peptides. Collagen, peptide solutions (0.1 mg/mL), and Boc derivatives (1 mM) were prepared in 0.1 M phosphate buffer (pH 7.4). These solutions were placed in fused quartz tubes at a distance of 7 cm from a low-pressure mercury lamp (Eiko Co.) without cooling systems and irradiated at room temperature.

Gel Filtration. An aliquot of an irradiated collagen solution was used for gel filtration, which was performed on TSK gel $G3000SW_{XL}$, 7.8 × 300 mm (Tosoh Co.). The column was equilibrated in 0.1 M phosphate buffer (pH 7.0), containing 0.1 M NaCl, and eluted by this buffer solution at a flow rate of 0.8 mL/min. The elution was monitored by UV absorbance at 215 nm.

Amino Acid Analysis. Amino acid analysis was performed with a JEOL JLC-300 amino acid analyzer. The samples were prepared as followed: After irradiation, the solutions were freeze-dried and then hydrolyzed with 6 N HCl in vacuo for 24 h at 110 °C. The hydrolysates were concentrated, dissolved in aqueous HCl (pH 2.2), and then submitted to the amino acid analyzer.

Gas Chromatography–Mass Spectrometry (GC–MS) of *N*-Heptafluorobutyl Isobutyl Ester Derivatives. Poly(L-pro-

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line) solution (0.1 mg/mL) was irradiated for 2 h. The solution (1 mL) was freeze-dried and then hydrolyzed with 6 N HCl in vacuo for 24 h at 110 °C. To remove inorganic salts, the hydrolysate was concentrated, dissolved in 1 mL of 0.1 N HCl, and then submitted for cation-exchange chromatography as described by Kaiser et al. (1974) with some modification. The solution was placed onto a 4.0-mL cation resin bed of Dowex 50W-X8 (H^+), 100–200 mesh, in a 12 × 35 mm column. The resin was washed with deionized water until the pH of the effluent was neutral. The hydrolysate was eluted from the column with a 40-mL volume of 7 N NH_4OH eluent. The eluate was collected and concentrated. The preparation of derivatives of *N*-heptafluorobutyl isobutyl ester was performed according to the method of Peace (1977). GC–MS was performed on a Hewlett-Packard Model 5890 gas chromatograph, interfaced to a JEOL JMS-DX705L mass spectrometer, using a 15-m fused silica DB-1 capillary column (J&W Scientific). The temperature program was follows: injector temperature, 220 °C; column temperature, 100 °C; ramp to 220 °C at 3 °C/min.

HPLC Determination of (Pro-Pro-Gly)$_{10}$. (Pro-Pro-Gly)$_{10}$ was determined by reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 250 mm) (Nomura Chemical Co., Ltd.). An aliquot of the irradiated solution was applied to the column equilibrated in 0.1% trifluoroacetic acid (TFA). The peptide was eluted with a linear gradient of acetonitrile (0.75%/min) at a flow rate of 0.8 mL/min, the elution being monitored by absorbance at 215 nm. The residual substrate was determined from the chromatographic peak height.

Determination of H_2O_2 by ABTS. The formation of hydrogen peroxide was determined by 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) according to the method of Childs et al. (1975). In a tube, 1 mL of ABTS solution (1 mg/mL), 3 mL of 0.1 M phosphate buffer (pH 6.0), and 1 mL of horseradish peroxidase solution (10 units/mL) were mixed. Irradiated solution (0.5 mL) was added to the tube, and the solution was held at 37 °C in a water bath for 15 min. The absorbance of the solution was measured at 414 nm on a Hitachi U-1100 spectrophotometer.

Preparation of Boc Derivatives. *N-tert*-Boc-L-proline was purchased from Sigma. *N-tert*-Boc-L-prolyl-glycine was prepared by *tert*-butoxycarbonylation of L-prolylglycine with *tert*-butyl *S*-(4,6-dimethylpyrimidin-2-yl)thiocarbonate according to the method of Nagasawa et al. (1973). The *N-tert*-Boc-L-prolylglycine was identified by amino acid analysis, FAB-MS, and ^1H NMR. The *tert*-butoxycarbonylation of 2-pyrrolidone with di-*tert*-butyl dicarbonate was performed as described by Grehn et al. (1986). The synthetic product was identified as *N-tert*-Boc-2-pyrrolidone by amino acid analysis, ^1H NMR, and ^{13}C NMR.

Isolation of the Oxidation Products Derived from *N-tert*-Boc-L-proline and *N-tert*-Boc-L-prolylglycine with Ultraviolet Irradiation. The peptide solution (1 mM) in 0.1 M phosphate buffer (pH 7.4) was irradiated for 2 (*N-tert*-Boc-L-proline) or 5 h (*N-tert*-Boc-L-prolylglycine) at room temperature. After irradiation, the solution was freeze-dried, extracted with methanol to remove a large quantity of the inorganic salts, and then evaporated in vacuo. The extract was dissolved in a TFA (0.1%)–methanol solution and then submitted to a Develosil ODS-10 column (20 × 250 mm). The products were eluted with a solution of TFA (0.1%)–methanol (5:4) for *N-tert*-Boc-L-proline and TFA (0.1%)–methanol (3:2) for *N-tert*-Boc-L-prolylglycine at a flow rate of 5 mL/min, and each peak was fractionated and further purified using a Develosil ODS-5 column (8 × 250 mm).

Instrumental Analysis. Fast atom bombardment mass spectrometry (FAB-MS) was performed with a JEOL JMS-DX705 mass spectrometer. The sample was dissolved in methanol and added to a drop of glycerol on the target.

Nuclear magnetic resonance (NMR) spectrometry on a JEOL JNX-200 spectrometer was taken in CD_3OD with tetramethylsilane as the internal standard.

RESULTS

Oxidative Degradation of Collagen during Ultraviolet Irradiation. Collagen solution (0.1 mg/mL) was irradiated with a low-pressure mercury lamp at room temperature. The time-dependent changes of collagen were examined by HPLC on TSK gel G3000SW$_{XL}$, and the elution pattern is shown in Figure 1. The high molecular weight parts of the collagens decreased and the low molecular products increased with irradiation. Oxidative degradation was presumed to be the predominant reaction in the system.

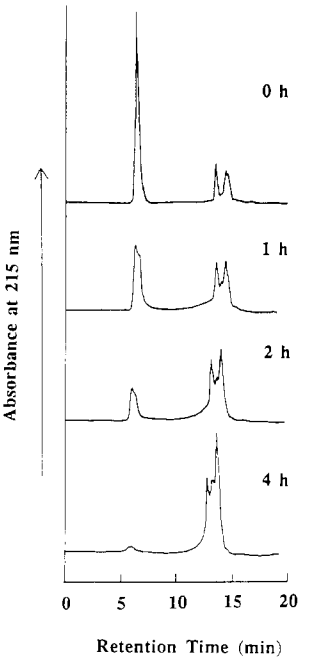

Figure 1. Time-dependent changes in the HPLC gel filtration chromatograms of collagen during irradiation by ultraviolet light.

Changes in Amino Acid Compositions of Collagen during Irradiation by Ultraviolet Light. To clarify the changes of amino acid residues in collagen, irradiated collagen was hydrolyzed with 6 N HCl and then submitted for amino acid analysis as described under Materials and Methods. As shown in Table I, large amounts of methionine, tyrosine, phenylalanine, and histidine decreased with ultraviolet irradiation on the basis of the change (percent). The result suggests that these changes occurred mainly in the side chain of these amino acids. Hydroxyproline and proline also decreased with irradiation by ultraviolet light. The decreases of the change (percent), which indicates the sensitivity of the amino acid to ultraviolet light, were not so specific for hydroxyproline (93.1%) and proline (88.9%). However, the differences of the concentration of hydroxyproline and proline were −52.5 and −121.1 nmol/mg of collagen, respectively. These large differences derived from not only the high sensitivity of proline and hydroxyproline but also the amino acid composition of collagen molecule because of large amounts of proline and hydroxyproline in the polypeptide chains. In view of the amounts, the large difference of proline concentration probably contributed to the degradation of collagen. Glutamic acid, aspartic acid, unknown product X, and ammonia increased with irradiation. The unknown product X was also detected in the hydrolysates of irradiated poly(L-proline) (see below). The result suggested that X was one of the oxidation products of proline and/or hydroxyproline.

Amino Acid Analysis of UV-Irradiated Poly(L-proline). Proline is one of the major components of collagen. It is assumed that the oxidation of proline is responsible for the oxidative cleavage of prolyl peptide (Uchida et al., 1990). Then poly(L-proline) was irradiated by ultraviolet light and hydrolyzed with 6 N HCl, and amino acid analysis

Table I. Changes in Amino Acid Compositions of Collagen during Irradiation of Ultraviolet Light[a]

amino acid	concn, of nmol/mg of protein 0 h	1 h	change,[b] %	diff[c]
hypro	756	704	93.1	-52
X[d]	0	154		154
Asp	354	375	105.9	21
Thr	156	150	96.3	-6
Ser	261	251	96.5	-10
Glu	569	582	102.3	13
Pro	1096	975	88.9	-121
Gly	2384	2348	98.5	-36
Ala	601	603	100.2	2
Cys	11	9	83.6	-2
Val	225	224	99.5	-1
Met	84	59	70.9	-25
Ile	157	142	90.2	-15
Leu	282	248	88.0	-34
Tyr	47	29	61.5	-18
Phe	134	73	54.7	-61
His	44	33	75.1	-11
hylys	271	234	86.2	-37
Lys	97	92	94.5	-5
ammonia	323	447	138.1	124
Arg	254	223	87.9	-31

[a] Collagen solution (0.1 mg/mL) was prepared in phosphate buffer (pH 7.4) and irradiated with a low-pressure mercury lamp at room temperature. [b] Change, percentage of the amino acid concentration of irradiated collagen per that of nonirradiated collagen. [c] Difference, subtraction of the amino acid concentration of nonirradiated collagen from that of irradiated collagen. [d] X is unknown product.

Table II. Amino Acid Analysis of Irradiated Poly(L-proline) by Ultraviolet Light[a]

amino acid	molar ratio,[b] % 0 h	1 h	2 h	4 h
hypro	0.00	0.14	0.23	0.44
X[c]	0.00	2.59	3.53	5.62
Asp	0.00	0.19	0.12	0.29
Ser	0.00	0.35	0.17	0.35
Glu	0.02	1.88	2.69	6.13
Pro	99.64	93.09	90.93	81.11
Gly	0.03	0.22	0.18	0.47
GABA[d]	0.00	0.59	0.92	1.98
ammonia	0.20	0.77	1.15	3.08

[a] Poly(L-proline) solution (0.1 mg/mL) was prepared in phosphate buffer (pH 7.4) and irradiated with a low-pressure mercury lamp at room temperature. [b] Molar ratio, molar concentration of each amino acid per total amino acids. [c] X is unknown product. [d] GABA, γ-aminobutyric acid.

was performed. The changes of the amino acid compositions are shown in Table II. UV-irradiated poly(L-proline) generated considerable amounts of glutamic acid, unknown product X, and ammonia in the acid hydrolysates. The unknown X was probably dehydroproline because of the same retention time as authentic dehydroproline. Poston (1976) identified *trans*-4-hydroxyproline, *cis*-4-hydroxyproline, and dehydroproline among the products of poly(L-proline) oxidation by an ascorbate/iron/EDTA system. We identified γ-aminobutyric acid (GABA) in the hydrolysates. The generation of significant amounts of GABA suggests that it is derived from 2-pyrrolidone by acid hydrolysis (Uchida et al., 1990). Further identification was performed by GC–MS as the *N*-heptafluorobutyl isobutyl ester derivatives of hydrolysates of UV-irradiated poly(L-proline). Total ion current chromatogram of the derivatives is shown in Figure 2. Peaks a–d were the same retention times as the standard derivatives of GABA, dehydroproline, proline, and glutamic acid, respectively. The mass chromatogram of peak a is shown in Figure 3. On the basis of the data, product a was identified as the *N*-heptafluorobutyl isobutyl ester derivative of GABA. The proposed structure is represented in Figure 3. The other peaks (b–d) provided the molecular ions m/z 365, 367, and 455, respectively. Peak b was assumed to be the derivative of dehydroproline on the basis of the retention time and the molecular ion (m/z 365). However, the amount of product b was very small on the basis of total ion current chromatogram as shown in Figure 2. In addition, the Boc derivative of neither dehydroproline nor the precursor, which is converted to dehydroproline by acid hydrolysis, was isolated from both UV-irradiated *N*-*tert*-Boc-L-proline and *N*-*tert*-L-prolylglycine (see Isolation and Characterization of the Degradation Products Derived from Irradiated Prolyl Peptide). Therefore, we are not very sure of the structure of X. Further identification of X must be performed in future. Products c and d were identified as the *N*-heptafluorobutyl isobutyl ester derivatives of proline and glutamic acid, respectively, on the basis of the mass chromatograms (data not shown).

Oxidative Degradation of (Pro-Pro-Gly)$_{10}$ during Ultraviolet Irradiation and Participation of Active Oxygen. A collagen molecule is characterized by a typical (X-Y-Gly) repeating sequence. X and Y are often hydroxyproline and proline, respectively. (Pro-Pro-Gly)$_{10}$ was used as a collagen model peptide, and then the oxidative degradation and the formation of GABA during ultraviolet irradiation were investigated. As shown in Figure 4A, a time-dependent loss of (Pro-Pro-Gly)$_{10}$ was observed. The irradiated peptide was hydrolyzed by 6 N HCl and then submitted for amino acid analysis. The formation of GABA was accompanied by the loss of proline residues (Figure 4B). Glutamic acid was also produced severalfold relative to GABA, and glycine was less reactive than proline (data not shown). Newly found products in the hydrolysates were all proline oxidation products, which were also detected in the hydrolysates of irradiated poly(L-proline).

It has been known that excitation of a peptide molecule by ultraviolet light caused oxidation of the peptide. However, the active oxygen as H_2O_2 and O_2^- can be formed from irradiated proteins (Andley and Clark, 1989). These active oxygen species are converted to HO$^\bullet$ or HOO$^\bullet$, which can attack biomolecules easily. To confirm the contribution of these oxidizing species for (Pro-Pro-Gly)$_{10}$ oxidation, the formation of H_2O_2 was determined according to the ABTS method. As shown in Figure 5, the generation of H_2O_2 from the irradiated solution was confirmed in the system. The formation was promoted in the presence of SOD, and no acceleration was observed in the presence of denatured SOD, which was autoclaved for 20 min at 120 °C. The result suggests that the enhancement was due to the enzyme activity of SOD, which catalyzed the dismutation of O_2^-. In addition, H_2O_2 was also detected in the irradiated solution of collagen, poly(L-proline), and other amino acid homopolymers (data not shown). Whitburn et al. (1982) reported the generation of H_2O_2 by γ-radiolysis of aqueous ferrimyoglobin solution. The reaction of the H_2O_2 formation is represented as

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

The reaction might occur in our system. However, the formation of H_2O_2 was inhibited under anaerobic condition (triangles, Figure 5). Most of H_2O_2 was probably generated from the disproportionation of O_2^- (see below). We also examined the effect of SOD (170 units/mL) on the degradation of the peptide by HPLC determination. In the absence of SOD, the residual was 48.4% with ultraviolet irradiation. The loss of (Pro-Pro-Gly)$_{10}$ was accelerated

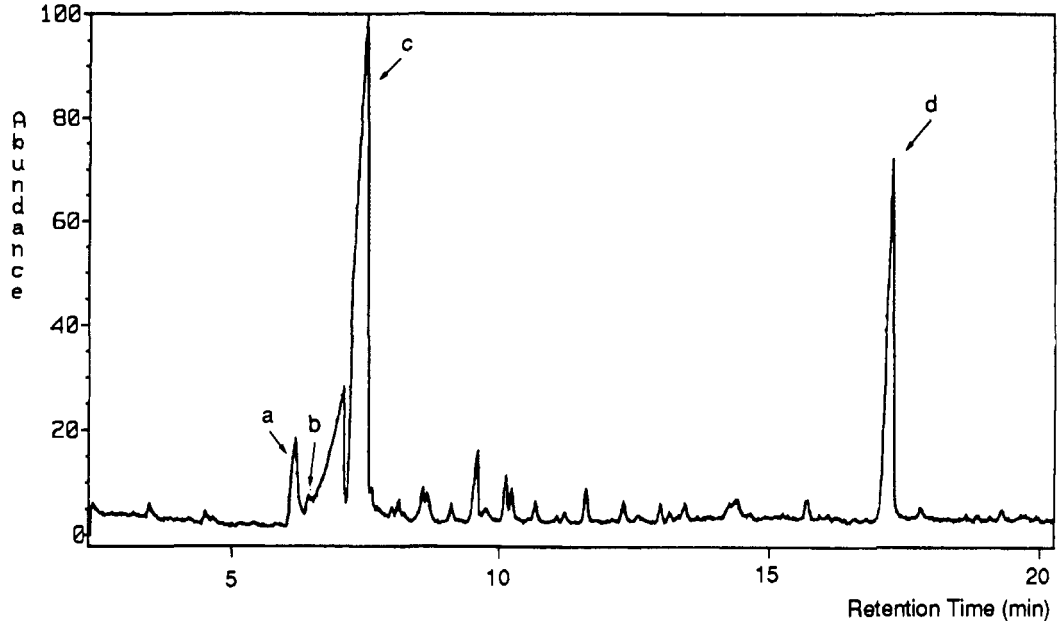

Figure 2. Total ion current chromatogram of *N*-heptafluorobutyl isobutyl ester derivatives of hydrolysates of irradiated poly(L-proline). The prolyl peptide was irradiated for 2 h and then hydrolyzed. The derivatization of the hydrolysates was performed as shown under Materials and Methods. The molecular ions are as follows: peak a, 355; peak b, 365; peak c, 367; peak d, 455.

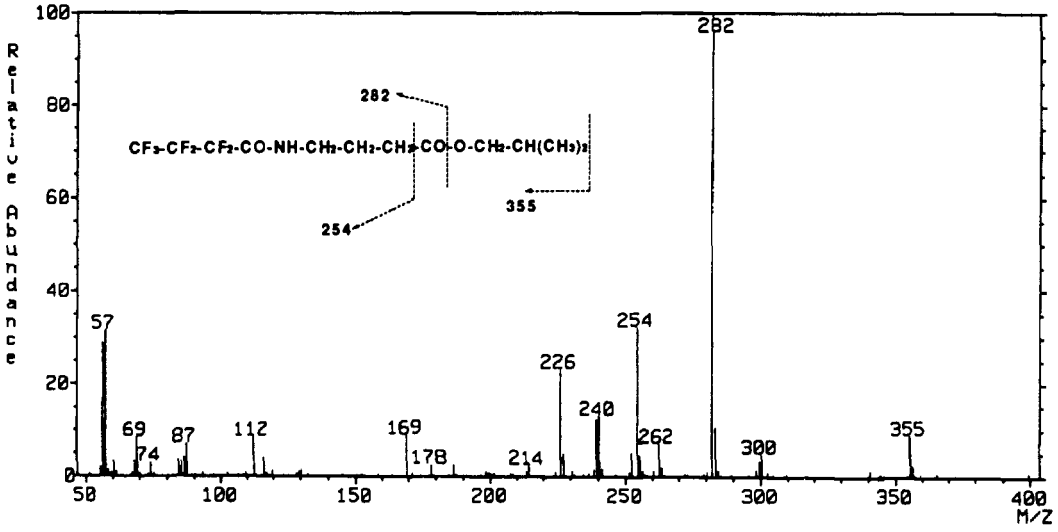

Figure 3. Mass chromatogram of peak a.

in the presence of SOD (residual 38.3%), but denatured SOD had no effect on the oxidation (residual 45.7%). The acceleration of loss of peptide by SOD may be due to consumption of O_2^-, which was generated by excitation of a charge-transfer (CT) complex consisting of an oxygen molecule and the peptide (Hori et al., 1970; Shizuka et al., 1972; Kulevsky et al., 1973). It is known that a hydroxyl radical is produced from the decomposition of H_2O_2 by ultraviolet light. Decomposition of H_2O_2, which was probably generated from disproportionation of O_2^-, contributed to the oxidation of peptide at least in part. Singlet oxygen, $^1O_2$, is known to be one of the active oxygen species. Merkel et al. (1983) reported that the existing time of $^1O_2$ in D_2O (20 μs) is longer than that in H_2O (2 μs). $(Pro\text{-}Pro\text{-}Gly)_{10}$ solutions were freeze-dried, diluted with D_2O, and then irradiated for 1 h. The percentages of the disappearance of the peptide were 29.5% (in H_2O) and 29.8% (in D_2O), respectively. If $^1O_2$ could contribute to the oxidation, the loss of peptide in D_2O would be more than that in H_2O. However, deuterium effect on the oxidation was hardly observed. This indicates that $^1O_2$ was a minor participant in the oxidation of prolyl peptide in the system. In addition, we found that $^1O_2$, which was produced by photosensitization of rose bengal, had no effect on poly(L-proline) (unpublished result). It is concluded that not only excitation of the peptide but also generation of H_2O_2 was probably responsible for the oxidation of peptide in the system.

Isolation and Characterization of the Degradation Products Derived from Irradiated Prolyl Peptide. To establish the oxidation mechanism of proline residue in a peptide chain, we examined the oxidation of *N-tert*-Boc-L-proline and *N-tert*-Boc-L-prolylglycine with ultraviolet irradiation. Two newly formed products were detected in each irradiated solution by HPLC (Figure 6). The oxidation products of *N-tert*-Boc-L-proline were named BP-1 and BP-2 (see Figure 6A). Those of *N-tert*-Boc-L-prolylglycine were named BPG-1 and BPG-2 (see Figure 6B). BP-2 and BPG-2 had the same retention times. *N-tert*-Boc-L-proline was not observed by HPLC in the irradiated *N-tert*-Boc-L-prolylglycine solution. The result indicates that no hydrolytic scission of peptide bond occurred. These products were isolated and characterized by amino acid analysis, FAB-MS, and ^1H NMR. The

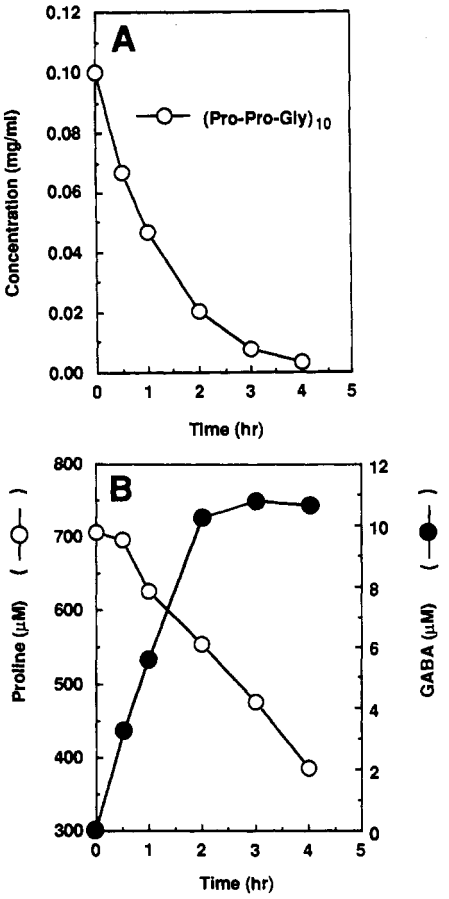

Figure 4. (A) Time-dependent disappearance of $(Pro\text{-}Pro\text{-}Gly)_{10}$ during ultraviolet irradiation. (B) Time-dependent loss of proline and production of GABA in the hydrolysates of $(Pro\text{-}Pro\text{-}Gly)_{10}$ solution during ultraviolet irradiation.

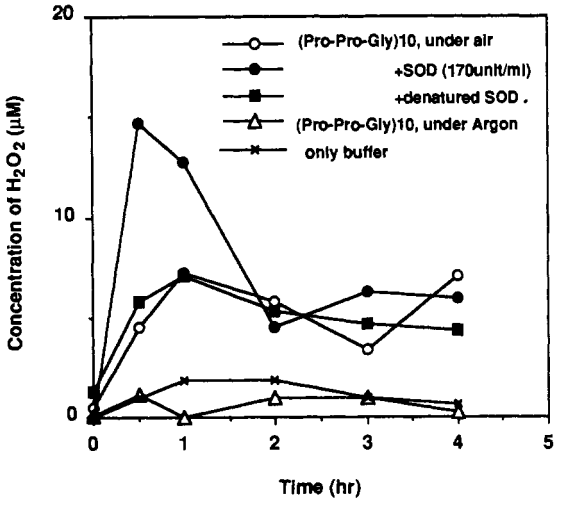

Figure 5. Formation of H_2O_2 from $(Pro\text{-}Pro\text{-}Gly)_{10}$ solution during ultraviolet irradiation.

proposed structures of the isolated products are summarized in Figure 7.

BP-1 was converted to glutamic acid by acid hydrolysis. The ^1H NMR spectrum of BP-1 revealed the disappearance of the C5 proton of proline. The ^1H NMR spectrum of authentic L-pyroglutamic acid was similar to that of BP-1. These results indicated that BP-1 should have the pyroglutamyl structure. BP-1 provided the $(M + H)^+$ ion, m/z 230, on the FAB-MS spectrum. On the basis of these data, BP-1 was identified as *N-tert*-Boc-L-pyroglutamic acid.

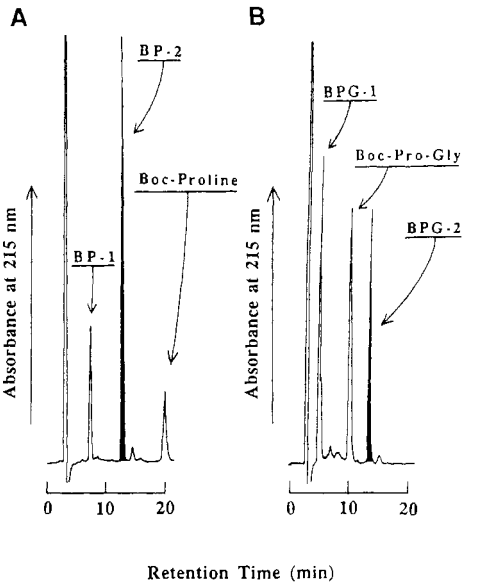

Figure 6. HPLC profiles of the oxidation products derived from *N-tert*-Boc-L-proline (A) and *N-tert*-Boc-L-prolyl-glycine (B) with ultraviolet irradiation for 120 min.

Identification of BPG-1 has been performed by amino acid analysis, ^1H NMR, and FAB-MS. Glutamic acid and glycine were detected by acid hydrolysis of BPG-1. The ^1H NMR spectrum of BPG-1 revealed the disappearance of the C5 proton of proline, suggesting the presence of a pyroglutamyl structure. The FAB-MS spectrum of BPG-1 gave a peak of the $(M + H)^+$ ion, m/z 287. Consequently, BPG-1 was identified as *N-tert*-Boc-L-pyroglutamylglycine.

Both BP-2 and BPG-2 provided GABA by their hydrolysis. We recently identified *N*-benzyloxycarbonyl (Z)-2-pyrrolidone as an oxidation product of Z-proline during incubation with H_2O_2 in the presence of copper(II) ion (Uchida et al., 1990). 2-Pyrrolidone is converted to GABA by hydrolysis. Then we presumed the presence of a 2-pyrrolidone structure in BP-2 and BPG-2. The ^1H NMR spectrum of the product was similar to those of Z-2-pyrrolidone and authentic 2-pyrrolidone. Therefore, the structure of the oxidation product was estimated as *N-tert*-Boc-2-pyrrolidone. Finally, the structures of BP-2 and BPG-2 were identified by direct comparison with synthetic *N-tert*-Boc-2-pyrrolidone, which was prepared by *tert*-butoxycarbonylation of 2-pyrrolidone with di-*tert*-dicarbonate. In addition, the $(M + H)^+$ ion of *N-tert*-Boc-2-pyrrolidone was not detected clearly on the FAB-MS spectrum.

Effects of Dissolved Oxygen on UV-Irradiated Peptide. To estimate the mechanism of fragmentation, the formation of *N-tert*-Boc-2-pyrrolidone from the irradiated *N-tert*-Boc-L-prolylglycine under air, nitrogen, and oxygen was determined by reverse-phase HPLC. As shown in Figure 8, a time-dependent loss of *N-tert*-Boc-L-prolylglycine occurred under aerobic conditions. In oxygenated solution, the loss of peptide was accelerated considerably. However, oxygen was not essential for the oxidative modification of peptide because the disappearance of *N-tert*-Boc-L-prolylglycine was observed under anaerobic condition. None of the new products other than BPG-1 and BPG-2 was observed from HPLC under anaerobic condtion (data not shown). The disappearance of the *N-tert*-Boc-L-prolylglycine under anaerobic condtion suggests that excitation of peptide contributed to the oxidative modification of *N-tert*-Boc-L-prolylglycine. The formation of *N-tert*-Boc-2-pyrrolidone was inhibited under nitrogen,

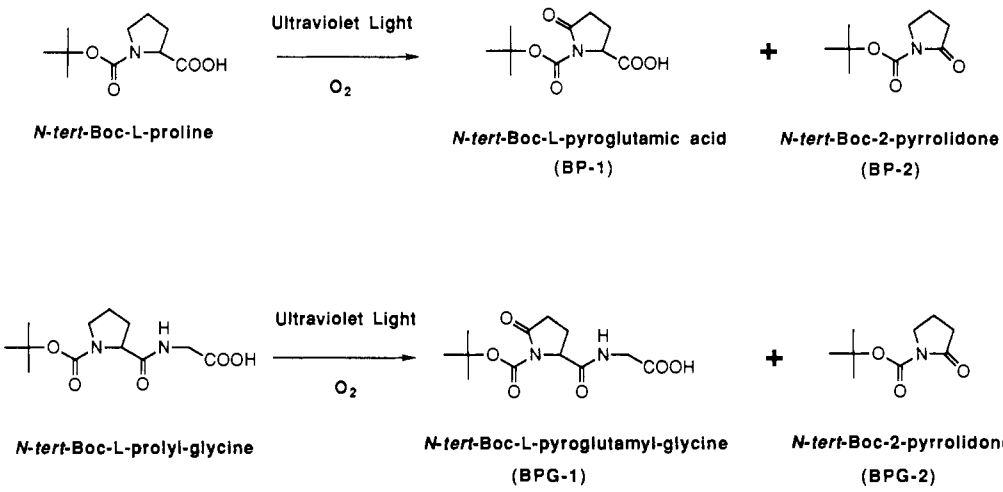

Figure 7. Proposed structures of isolated products. Structural data of the products are as follows. BP-1: ^{1}H NMR δ 1.32–1.55 (9 H, *tert*-butyl), 1.99–2.74 (4 H, CH_2CH_2), 4.19–4.28 (1 H, NCH); BP-1 was converted to glutamic acid by acid hydrolysis; FAB-MS 230 (M + 1). BPG-1: ^{1}H NMR δ 1.32–1.55 (9 H, *tert*-butyl), 1.98–2.74 (4 H, CH_2CH_2), 3.81–4.11 (2 H, CH_2), 4.80–4.85 (1 H, NCH); BPG-1 was converted to both glutamic acid and glycine by acid hydrolysis; FAB-MS 287 (M + 1). BP-2 and BPG-2: ^{1}H NMR δ 1.43–1.50 (9 H, *tert*-butyl), 1.93–2.09 (2 H, CH_2), 2.46–2.56 (2 H, CH_2), 3.70–3.81 (2 H, CH_2); BP-2 and BPG-2 were converted to γ-aminobutyric acid (GABA) by acid hydrolysis.

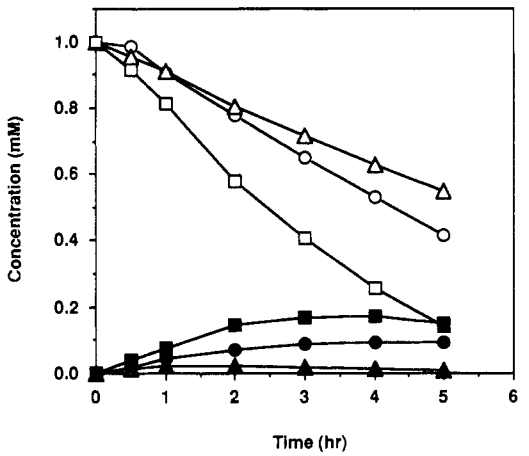

Figure 8. Time course of the disappearance of *N-tert*-Boc-L-prolylglycine and the production of *N-tert*-Boc-2-pyrrolidone during irradiation by ultraviolet light under various oxygen atmospheric conditions. The determination of *N-tert*-Boc-L-prolylglycine and *N-tert*-Boc-2-pyrrolidone were performed by reverse-phase HPLC on Develosil ODS-5 (8 × 250 mm). Saturation of O_2 and N_2 was performed by bubbling the gases in for at least 5 min. (○) *N-tert*-Boc-L-prolylglycine under air; (□) *N-tert*-Boc-L-prolylglycine under O_2; (△) *N-tert*-Boc-L-prolylglycine under N_2; (●) *N-tert*-Boc-2-pyrrolidone under air; (■) *N-tert*-Boc-2-pyrrolidone under O_2; (▲) *N-tert*-Boc-2-pyrrolidone under N_2.

although the substrate, *N-tert*-Boc-L-prolylglycine, was degraded in deoxygenated solution. The result indicates that oxygen is essential for the formation of *N-tert*-Boc-2-pyrrolidone, and therefore the oxygen atom, which is incorporated at the C2 carbon, must be derived from dissolved oxygen in aqueous solution.

DISCUSSION

Many studies on the fragmentation of collagen with ultraviolet irradiation have been performed (Cooper and Davidson, 1965, 1966; Davidson and Cooper, 1967; Miyata et al., 1971). However, there are very few works on the mechanism of its oxidative fragmentation of collagen. It has been known that degradation of collagen easily arises with various oxidation systems (Curran et al., 1984; Monboisse et al., 1983; Bowes and Moss, 1962). We also found the fragmentation of collagen and degradation of its proline residue with ultraviolet irradiation. It was presumed that its fragmentation depended on the oxidative degradation of the proline residue. Then, poly(L-proline) and $(Pro\text{-}Pro\text{-}Gly)_{10}$ as collagen models were used, and the oxidation of model peptides by ultraviolet irradiation was investigated in detail. GABA was found in the hydrolysates of the irradiated peptides. Recently, it has been considered that the formation of GABA indicated oxidative fragmentation of peptide containing proline. The formation of 2-pyrrolidone, which is one of the oxidation products of proline and converts to GABA by acid hydrolysis, probably contributes to cleavage of the peptide chain containing the proline residue (Uchida et al., 1990). In this study, we isolated *N*-tert-Boc-2-pyrrolidone from irradiated *N*-tert-Boc-L-prolylglycine solution. The identification of the pyrrolidone compound from the dipeptide was direct evidence that the formation of the pyrrolidone compound contributed to the fragmentation of prolyl peptide on the basis of its structural property. However, GABA from the hydrolysates of irradiated collagen was undetected because GABA has the same retention time as phenylalanine on amino acid analysis.

We determined the formation of H_2O_2 from the irradiated poly(L-proline) solution. SOD accelerated both the generation of H_2O_2 and the loss of $(Pro\text{-}Pro\text{-}Gly)_{10}$ by ultraviolet irradiation. It is known that $^1O_2$ often plays important roles in photosensitized oxidation. However, $^1O_2$ was not a participant in the oxidation of prolyl peptide. These results suggest that active oxygen species, such as hydroxyl radical or O_2^-, except $^1O_2$, contributed to some extent to these oxidations.

The oxygen atom of 2-pyrrolidone would be incorporated at the C2 carbon from the dissolved oxygen in the medium because the formation of the 2-pyrrolidone compound was accelerated in oxygenated solution and depressed significantly in deoxygenated solution (Figure 8). The proposed mechanism for photoinduced fragmentation of peptide containing proline residue is summarized in Figure 9.

In this work, the formation of the 2-pyrrolidone compound, which indicated the scission of the peptide chain at the position of the proline residue, was confirmed in photooxidation of model peptides of a collagen. 2-Pyrrolidone was also produced by the Fenton reaction (Uchida et al., 1990) and γ-irradiation (unpublished result). The 2-pyrrolidone structure and GABA, which is a hydroly-

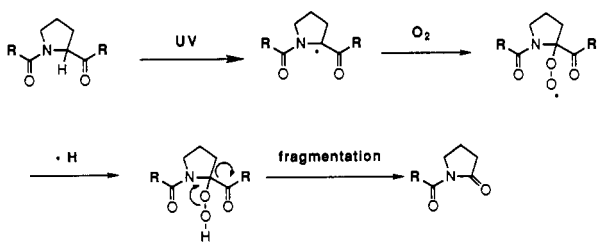

Figure 9. Proposed mechanism for oxidative cleavage of prolyl peptide.

sate of 2-pyrrolidone, may become an indicator of not only food deterioration but also aging under various oxidative stresses.

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Received for review September 4, 1991. Revised manuscript received December 6, 1991. Accepted December 20, 1991.

Registry No. GABA, 56-12-2; BPG-2, 85909-08-6; BPG-1, 138813-35-1; O_2^-, 11062-77-4; H_2O_2, 7722-84-1; OH·, 3352-57-6; OOH·, 3170-83-0; $(Pro\text{-}Pro\text{-}Gly)_{10}$, 22967-48-2; poly(L-proline), 25191-13-3; poly(L-proline), SRU, 25213-33-6; ammonia, 7664-41-7; *N-tert*-Boc-L-proline, 15761-39-4; *N-tert*-Boc-L-prolylglycine, 51785-82-1; glutamic acid, 56-86-0; *N-tert*-Boc-L-pyroglutamic acid, 53100-44-0.