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A NOVEL MECHANISM FOR OXIDATIVE CLEAVAGE OF PROLYL PEPTIDES INDUCED BY THE HYDROXYL RADICAL

Koji Uchida[1], Yoji Kato, and Shunro Kawakishi

Laboratory of Chemistry of Plant Products, Department of Food Science & Technology, Nagoya University, Nagoya 464-01, Japan

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SUMMARY: Mechanism for the oxidative cleavage of proline-containing peptides induced by the hydroxyl radical (·OH) has been studied. Accompanying the oxidation of prolyl peptides, we discovered the formation of significant amounts of γ-aminobutyric acid (GABA) in the acid hydrolysates of the oxidized peptides. GABA was assumed to be derived from the 2-pyrrolidone compound and, in addition, its generation led to the assumption that prolyl peptides were mainly cleaved at the proline residues by ·OH, accompanied by the oxidative modification of proline by itself. Hence, in order to confirm this prediction, we undertook the reaction of proline with ·OH using proline analogue (Z-proline) and isolated the 2-pyrrolidone compound as the major product. We proposed a novel mechanism for formation of the 2-pyrrolidone compound induced by ·OH, which has been established for the first time in the oxidative cleavage of prolyl peptides.

Formation of highly reactive free radicals such as superoxide (O_2^-) or hydroxyl radicals (·OH) occurs in many important pathologic processes such as oxygen and other gaseous toxicity, microbial killing by phagocytic cells, inflammatory damage, and endotherial injury (1-5). The precise mechanisms of oxygen-mediated cell and tissue injury are not well known; however, several studies have suggested that oxygen-free radicals, especially ·OH, have a direct effect on destruction of tissue components such as proteins (6-9).

Previous studies of free radical damage to proteins have indicated that a wide variety of reactions can occur (10-13). We also have investigated the oxidative modification of serum albumin with the metal-

[1] To whom correspondence should be addressed.

Abbreviations: Z, Nα-benzyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; EI-MS, electron impact-mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; NMR, nuclear magnetic resonance; GABA, γ-aminobutyric acid.

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catalyzed free radical systems such as a metal/ascorbate system (14,15) and a metal/H_2O_2 system (16). In addition, we have so far established novel reactions onto histidine- and tryptophan-residues analogues mediated by these systems (17-19). Whereas, we have concentrated our efforts on proline-containing polypeptides such as collagen because of its characteristic compositional features (20).

In the present communication, we have discovered a novel ·OH-mediated reaction onto proline residues in prolyl peptides and have established a mechanism which represents the oxidative cleavage of proline-containing peptides or proteins by ·OH.

MATERIALS AND METHODS

Materials

Poly-L-proline (Mol.Wt. 10^3-10^4), and poly(Pro-Gly-Pro) (Mol.Wt. $2x10^3$-10^4) were purchased from Sigma. Z-Proline, Z-Gly-Pro-Leu-Gly-Pro, $(Pro-Gly-Pro)_5$, and $(Pro-Gly-Pro)_{10}$ were obtained from Peptide Institute Inc. Ethylenediaminetetraacetic acid (EDTA) disodium salt, $CuSO_4 \cdot 5H_2O$, and 2-pyrrolidone 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 cleavage of prolyl peptides

Solutions containing 0.5 mg polyproline, poly(Pro-Gly-Pro), $(Pro-Gly-Pro)_{10}$, or $(Pro-Gly-Pro)_5$ were exposed to 5 mM H_2O_2 in the presence of 0.05 mM copper(II) ion, in a total volume of 1 ml of 0.1 M sodium phosphate buffer, pH 7.4. The reaction mixtures were incubated at 37 °C. The reactions were initiated by the addition of H_2O_2 and stopped by the addition of 0.1 mM EDTA. After incubations, the reaction mixtures were freeze-dried and then submitted for amino acid analysis.

Oxidation of a prolyl analogue with copper(II)/hydrogen peroxide

Solution containing 1 mM Z-proline was exposed to 5 mM H_2O_2 in the presence of 0.05 mM copper(II) ion, in a total volume of 1 ml of 0.1 M sodium phosphate buffer, pH 7.4. The reaction mixture was incubated at 37 °C. The reaction was initiated by the addition of H_2O_2 and stopped by the addition of 0.1 mM EDTA.

Both substrate and products in the reaction mixtures were determined by reverse-phase HPLC on a Develosil ODS-5 column (10 x 250 mm). Z-Proline was eluted with 50% methanol in 0.1% trifluoroacetic acid at a flow rate of 2.5 ml/min, the elution being monitored by absorbance at 210 nm. Areas of the chromatographic peaks of each material were calculated using a Shimadzu Chromatopac Integrator, Model C-R3A.

Amino acid composition

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: the collagen and other prolylpeptide samples exposed to 5 mM H_2O_2 and 0.05 mM $CuSO_4$ for 24 h 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 for amino acid analysis.

Mass Spectrometry

Electron impact-mass spectrometry (EI-MS) and fast atom bombardment-mass spectrometry (FAB-MS) were performed with a JEOL JMS-DX705 mass spectrometer. For FAB-MS, the sample was dissolved in glycerol and 1 nmol in 0.5 μl of matrix was deposited on a stainless steel probe tip and placed in the ion source where it was bombarded with a beam of xenon atoms from a JEOL neutral atom gun (5 KeV, 2 A cathode current, 10 mA emission).

Nuclear Magnetic Resonance Spectrometry

A nuclear magnetic resonance (NMR) spectrum on a JEOL JNM-FX200 spectrometer was taken in acetone-d_6 with tetramethylsilane as the internal standard.

RESULTS

Oxidative cleavage of prolyl peptides

It has been characterized that collagen is sensitive to oxidation and is degraded by active oxygen species such as O_2^-, ozone, or ·OH (21,22).

TABLE I

Oxidation of Prolyl Peptides upon Incubation with Hydrogen Peroxide in the Presence of Copper(II) Ion

Substrate	Molar ratio (%)				
	Pro	Gly	Glu	Hypro	GABA
Z-Gly-Pro-Leu-Gly-Pro					
native	45.11	35.42	--	--	--
oxidized	42.53	33.84	1.21	0.37	0.51
$(Pro-Gly-Pro)_5$					
native	74.15	25.37	--	--	--
oxidized	56.90	26.05	5.15	4.75	1.76
$(Pro-Gly-Pro)_{10}$					
native	74.15	25.24	--	--	--
oxidized	58.07	25.25	5.33	4.87	1.65
Poly(Pro-Gly-Pro)					
native	73.14	25.25	0.26	0.04	0.29
oxidized	57.13	25.02	4.56	4.03	1.71
Poly-L-proline					
native	99.38	--	--	--	0.01
oxidized	80.84	--	4.11	10.03	0.91

Molar ratio (%) was represented by the mole concentration of each amino acid per total amino acid. Hypro and GABA represent hydroxyproline and γ-aminobutyric acid, respectively.

We also have characterized the depolymerization of collagen upon incubation with H_2O_2 in the presence of copper(II) ion, accompanied by selective damage of proline residues (Uchida et al., unpublished results). We therefore speculated that oxidative damage to proline correlates to the depolymerization of collagen by itself.

Hence, we attempted to characterize the mechanism of oxidation of proline followed by oxidative cleavage of collagen, using various prolyl peptides, poly-L-proline, poly(Pro-Gly-Pro), $(Pro\text{-}Gly\text{-}Pro)_{10}$, and $(Pro\text{-}Gly\text{-}Pro)_5$, as the substrates. Reaction of these prolyl peptides with $Cu(II)/H_2O_2$ was assessed by the loss of proline and by the concomitant formation of oxidized products. As shown in Table I, prolyl peptides exposed to $Cu(II)/H_2O_2$ generated considerable amounts of hydroxyproline and glutamic acid in the acid hydrolysates of oxidized peptides. In addition, we discovered the generation of significant amounts of γ-aminobutyric acid (GABA), which was assumed to be derived from the acid hydrolysis of the oxidized proline. At this stage, we considered the possibility that GABA arised from a 2-pyrrolidone compound which directly correlates to the oxidative cleavage of these peptides.

Isolation and characterization of the 2-pyrrolidone compound

In order to characterize the 2-pyrrolidone compound in the ·OH-mediated oxidation of prolyl compounds chemically, the reaction of prolyl analogues with $Cu(II)/H_2O_2$ was undertaken using Z-proline as the substrate. Figure 1 represents the HPLC profile of the reaction mixture after 24 h of incubation. The reaction of the proline derivative with $Cu(II)/H_2O_2$ provided three products (**a** - **c**), selectively.

Finally, the product corresponding to the 2-pyrrolidone compound was found to be the product **c**. Among these products, only **c** provided GABA after acid hydrolysis, suggesting the presence of the 2-pyrrolidone structure. Product **c** provided the molecular ion, m/z 219, on the EI-MS and the quasimolecular ion, m/z 220 (M+1), on the FAB-MS spectrum. The ^1H-NMR spectrum of **c** revealed the disappearance of the α-CH proton and a shift

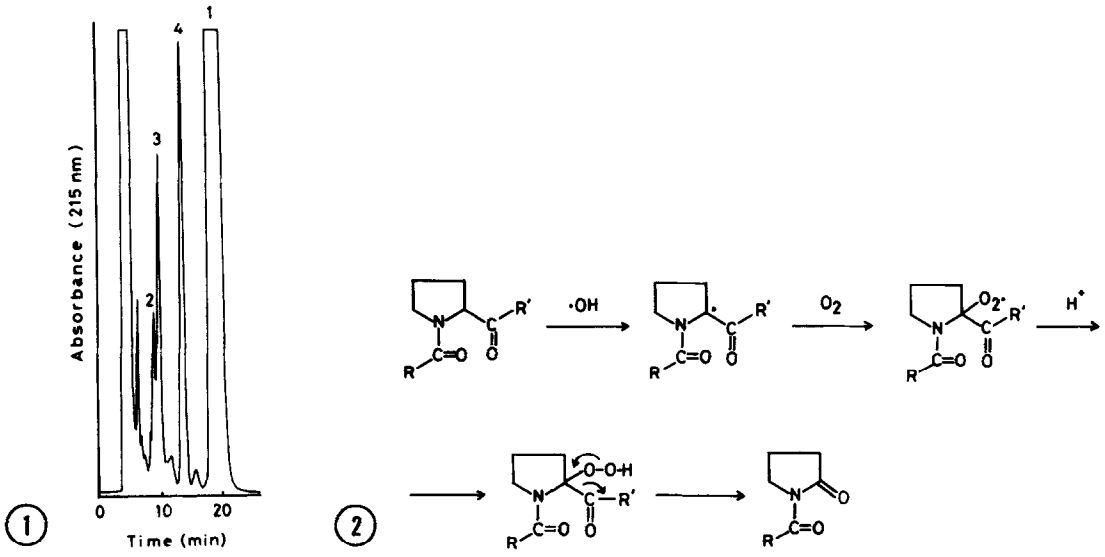

Fig. 1. HPLC profile of the oxidized proline derivative. Reaction mixture in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM Z-proline, 0.05 mM copper(II) ion, and 5 mM H_2O_2 was incubated for 24 h at 37°C. Peak numbers 1, 2, 3, and 4 represent Z-proline, product **a**, product **b**, and product **c**, respectively.

Fig. 2. A proposed mechanism for oxidative cleavage of prolyl peptides induced by ·OH.

of the C-3 methylene protons from 3.55 ppm to 3.84 ppm. In addition, the ^{1}H-NMR spectrum of the authentic 2-pyrrolidone fully supported the presence of 2-pyrrolidone structure within the product **c**. These results indicate that product c should have the 2-pyrrolidone structure. Whereas, products **a** and **b** were identified as Z-pyroglutamic acid and hydroxy-Z-proline, respectively.

A proposed mechanism for generation of 2-pyrrolidone compound by ·OH is summarized in Fig. 2. The hydroxyl radical-mediated hydrogen abstraction occurs at the α-carbon leading, via a peroxy radical, to a 2-pyrrolidone compound.

DISCUSSION

Our proposed mechanism accounts for the generation of the 2-pyrrolidone compound as the new C-termini and predicts the generation of new N-terminal amino acid residue. In relation to this, we have recently characterized that N-amino terminal sequence analysis of the oxidized

collagen with ·OH clearly demonstrates the appearance of a considerable amount of glycine (Uchida et al., unpublished result). We supposed that selective generation of glycine as the N-terminal amino acid represented the selective damage to the Y residue in Gly-X-Y repeating sequence of collagen. Therefore, at present, we speculate the mechanism for oxidative cleavage of Pro-Gly peptide bond in collagen to generate the 2-pyrrolidone compound as the C-termini and glycine residue as the N-termini (Fig. 3).

On the other hand, glutamic acid was identified as the major product in acid hydrolysates of prolyl peptides, following their oxidation with ·OH (Table I). While, it is apparent that conversion of proline to other amino acids such as hydroxyproline and pyroglutamic acid does not correlate to the oxidative scission of polypeptide chains by themselves. Therefore, we consider that the oxidative cleavage of collagen and other prolyl polypeptides is responsible for the oxidation of proline residues accompanied by the generation of the 2-pyrrolidone structure.

In this study, we have established a novel mechanism for oxidative cleavage of prolyl peptides, in which the formation of the free radical give rise not only to the oxidative modification of proline residues but to the oxidative cleavage of prolyl peptide bonds. We believe that our results represent the novel mechanism of aging mediated by free radicals in biological systems. Yet these propositions are speculation that will be addressed experimentally.

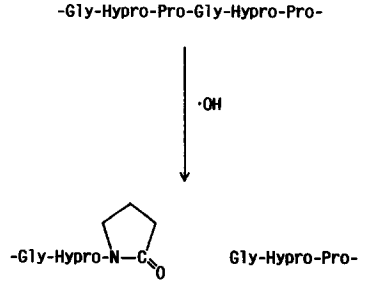

Fig. 3. A proposed mechanism for oxidative cleavage of collagen induced by ·OH.

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