Sequence-Dependent Reactivity of Histidine-Containing Peptides with Copper(II)/Ascorbate

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Copper(II)/ascorbate-mediated oxidative damage to the peptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe was accompanied by the loss of Asp (64%), Arg (49%), Val (35%), and His (52%). In addition, the reaction of copper(II)/ascorbate with the peptide gave three products (AII-1, AII-2, and AII-3). AII-1 was confirmed to be the product of oxidized His, which generated the 2-imidazolone structure. The damage of the N-terminal sequence was reflected by AII-2 and AII-3. Hence, we characterized the reactivity of the N-terminal sequence using Asp-Arg, Arg-Val, Val-Tyr, and Asp-Arg-Val-Tyr. The reactivities of preferred sequences were Asp-Arg-Val-Tyr > Asp-Arg > Arg-Val > Val-Tyr, suggesting that the Asp-Arg-Val-Tyr sequence is important for the reactivity with Cu(II)/ascorbate. On the other hand, the peptide Ile-His-Pro-Phe, corresponding to the C terminus of the native peptide, failed to react with Cu(II)/ascorbate, suggesting that the Asp-Arg-Val-Tyr sequence contributes to the reactivity of His with copper(II)/ascorbate.

INTRODUCTION

The acceleration of ascorbate autoxidation by metal ion is well-known. It is accompanied by the one-electron reduction of molecular oxygen with the reduced metal ion yielding oxygen-derived free radical species (Khan and Martell, 1967a,b). The metal/ascorbate system enhances the oxidation of various food and biological materials including proteins (Samuni et al., 1983; Shinhar et al., 1983; Marx and Chevion, 1985), which directly results in the fragmentation of protein and the oxidative modification of amino acid residues (Uchida and Kawakishi, 1988). In addition, metal-catalyzed protein oxidations have been recently implicated to be important causative agents of oxygen toxicity in aging and its related deseases (Oliver et al., 1987). Of particular interest is the fact that active species generated by Cu(II)/ascorbate cause a site-specific modification of proteins in which copper ions are bound to proteins. The nature of oxidized protein has not been characterized chemically; however, we have found the specific loss of the histidine residue in serum albumin to be the most characteristic change in the primary structure of the protein (Uchida and Kawakishi, 1988). Moreover, we have investigated the reaction of histidine with copper(II)/ascorbate using several histidine analogues, and a novel mono-oxygenation reaction of the imidazole ring has been established (Uchida and Kawakishi, 1986, 1989a, 1990a-c).

Meanwhile, we have recently found that the sequence of peptide significantly affects its reactivity with Cu(II)/ascorbate. To our knowledge, the sequence-specific reactivity has not been characterized in peptides or in proteins. Thus, the present work was undertaken to characterize the sequence of peptide susceptible to modification with Cu(II)/ascorbate using Asp-Arg-Val-Tyr-Ile-His-Pro-Phe as the substrate. In the course of this study, we found that Cu(II)/ascorbate caused selective damage to both the N-terminal region and a histidine residue of the peptide accompanied by the selective formation of three products. In addition, the N-terminal region was found to contribute significantly to the reaction of His with Cu(II)/ascorbate. In the present investigation, we have chemically characterized the products and found that the reactivity of a peptide with the copper(II)-dependent redox system was apparently dependent on its sequence.

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MATERIALS AND METHODS

Materials. Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II) acetate salt and Arg-Val were obtained from Sigma Chemical Co. L-Ascorbate, $CuSO_4 \cdot 5H_2O$, and ethylenediaminetetraacetic acid (EDTA) disodium salt were purchased from Wako Pure Chemical Industries, Ltd. Thermolysin (product from Seikagaku Kogyo Co., Ltd.) was a kind gift from the Laboratory of Chemistry of Animal Products, Department of Food Science and Technology, Nagoya University. Trifluoroacetic acid (TFA, >99%) was purchased from Tokyo Kasei Kogyo Co., Ltd. Other reagents were of the highest grades commercially available.

Reaction of the Peptide with Copper(II)/Ascorbate. Reactions were carried out at room temperature. The solution (2 mL) contained 0.5 mg of peptide, 5 mM ascorbate, and 0.05 mM $CuSO_4$ in 0.1 M sodium phosphate buffer (pH 7.4). Reactions were initiated by the addition of ascorbate and stopped by the addition of EDTA solution (0.05 mM).

The peptide was assayed by reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 250 mm). The reaction mixture was applied to a column equilibrated with a solution of a aqueous 0.1% trifluoroacetic acid. The peptide was eluted with a linear gradient of methanol (2.5%/min) at a flow rate of 0.8 mL/min, the elution being monitored by absorbance at 210 nm. Areas under the chromatographic peaks for each material were calculated using a Shimadzu Chromatopac integrator, Model C-R3A.

Thermolysin Digestion. The peptides Asp-Arg, Val-Tyr, Asp-Arg-Val-Tyr, and Ile-His-Pro-Phe were prepared by the enzymatic digestion of angiotensin II with thermolysin. Angiotensin II (10 mg) was dissolved with 10 mL of 0.1 M Tris-HCl (pH 8.0). One milliliter of 0.01% thermolysin in 0.1 M Tris-HCl (pH 8.0) containing 0.01 M $CaCl_2$ was then added to the peptide solution followed by incubation for 2 h at 37 °C. The mixture was freeze-dried and then applied to reverse-phase HPLC using a Develosil ODS-10 column (10 × 250 mm) equilibrated as above. The peptide was eluted with a linear gradient from 0.1% trifluoroacetic acid to methanol (2.5%/min) at a flow rate of 2.5 mL/min, the elution being monitored by absorbance at 210 nm. Finally, we obtained four peptides, Asp-Arg, Val-Tyr, Asp-Arg-Val-Tyr, and Ile-His-Pro-Phe, from angiotensin II. The sequence of each peptide was characterized by amino acid analysis of their acid hydrolysates on a JEOL JLC-300 amino acid analyzer equipped with a JEOL LC 30-DK20 data analyzing system.

Subsequently, the peptides Asp-Arg, Val-Tyr, Arg-Val, Asp-Arg-Val-Tyr, and Ile-His-Pro-Phe (0.5 mM) were exposed to 0.05

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mM Cu(II) and 5 mM ascorbate in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. The reactivity of peptides with Cu(II)/ascorbate was determined by reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 250 mm). The peptides were eluted with a linear gradient from 0.1% trifluoroacetic acid to methanol (2.5%/min) at a flow rate of 0.8 mL/min, the elution being monitored by absorbance at 210 nm.

Isolation and Identification of the Modified Peptides. The reaction mixture (10 mL) in 0.1 M sodium phosphate buffer (pH 7.4) containing 5 mM angiotensin II, 0.05 mM $CuSO_4$, and 5 mM ascorbate was incubated for 6 h at room temperature. Both substrate and products in the reaction mixture were monitored by HPLC at various time intervals. After incubation, the mixture was freeze-dried, dissolved in a small amount of distilled water, and then subjected to preparative HPLC on a Develosil ODS-10 column (20 × 250 mm). The products were eluted isocratically with a solution of 50% methanol in aqueous 0.1% trifluoroacetic acid at a flow rate of 6 mL/min. Products were detected by absorbance at 210 nm. Purification was achieved by multiple injection/peak collisions using a Develosil ODS-5 column (4.6 × 250 mm).

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. The reaction mixture, sampled at each time point, was freeze-dried and then hydrolyzed with 6 N HCl at a concentration of 1 mg of peptide/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. Similarly, modified peptides (50 μg), AII-1, AII-2, and AII-3, were hydrolyzed with 6 N HCl (50 μL) for 20 h at 110 °C 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 isolated peptide samples (approximately 1.0 μg of peptide in 30 μL of methanol) were 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 phenylthiohydantoin derivatives and injected into the on-line analyzer for identification.

Fast Atom Bombardment Mass Spectrometry. The fast atom bombardment mass spectrometry (FAB-MS) was performed with a JEOL JMS-DX705 mass spectrometer. 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 CD_3OD with tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

The relationship between the reactivity of histidine-containing peptides with Cu(II)/ascorbate and the loss of histidine residue is summarized in Table I. Most of the His-containing materials were reactive with Cu(II)/ascorbate, and the loss of substrates was completely dependent on the loss of histidine residue. Whereas in the cases of Asp-Arg-Val-Tyr-Ile-His-Pro-Phe and Arg-Val-Tyr-Ile-His-Pro-Phe, almost half of their reactivity can be explained by the loss of histidine, they apparently have other target molecules in the peptides. Hence, we focused on the reaction of Asp-Arg-Val-Tyr-Ile-His-Pro-Phe with Cu(II)/ascorbate.

As shown in Figure 1, the addition of 5 mM ascorbate and 0.05 mM copper(II) ion induced a rapid loss of the peptide after 4 h of incubation. We then characterized the change in amino acid composition of the peptide during incubation with Cu(II)/ascorbate. Figure 2 represents the comparison of the extent of modification of the peptide

Table I. Reactivity of Histidine Residue of Peptides with Copper(II) and Ascorbate[a]

peptide	incubation, h	loss of substrate, %	loss of His, %	*H/S*, mol/mol
His	24	82.9	82.9	1.00
Bz-His	24	85.0	85.0	1.00
His-Phe	24	77.0	77.5	1.01
His-Tyr	24	75.6	75.4	1.00
Gly-Gly-His	24	72.5	73.5	1.01
Bz-Gly-His-Leu	24	77.6	66.3	0.85
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	4	70.6	32.9	0.93
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	2	84.1	45.2	0.54
Arg-Val-Tyr-Ile-His-Pro-Phe	2	71.3	31.8	0.45

[a] The reactions were carried out a room temperature. The solution contained 1 mM substrate, 5 mM ascorbate, and 50 μM copper(II) ion in 0.1 M sodium phosphate buffer (pH 7.4). *H/S* represents the ratio of the loss of histidine residue (mol) per loss of the substrate (mol).

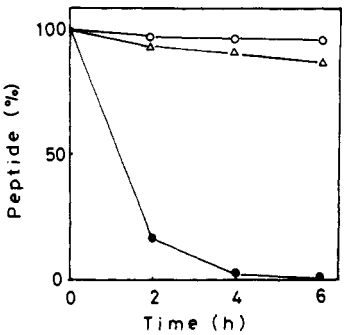

Figure 1. Time-dependent loss of a peptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) during incubation with the Cu(II)/ascorbate system. The reaction mixture (2 mL) in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.5 mg of peptide and 0.05 mM copper(II) ion (○), 5 mM ascorbate (△), or 0.05 mM copper(II) ion plus 5 mM ascorbate (●) was incubated at room temperature.

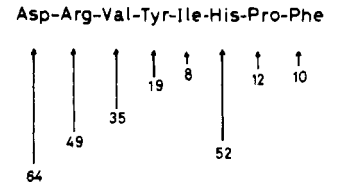

Figure 2. Comparison of the extent of reactivity of the peptide with copper(II)/ascorbate. Figures represent the loss of each amino acid after 4 h of incubation.

after 4 h of incubation. The result clearly indicates the presence of two separate target regions within a peptide; they are His and the N-terminal sequence (Asp-Arg-Val). It can be presumed that the intensive reactivity of this peptide with Cu(II)/ascorbate is based on the presence of two kinds of target regions.

On the other hand, the reaction of the peptide with Cu(II)/ascorbate gave three products (AII-1, AII-2, and AII-3) selectively (Figure 3). To characterize the oxidized peptides (AII-1, AII-2, and AII-3), isolation by preparative HPLC was undertaken. They were finally purified by reverse-phase HPLC using a Develosil ODS-5 column (4.6 × 250 mm). Yields of the products were approximately 160 μg of AII-1, 250 μg of AII-2, and 50 μg of AII-3. As shown in Table II, the amino acid analysis of the hydrolysates of the products clearly indicates that AII-1, AII-2, and AII-3 were oxidized products of His, of the N-terminal region, and of both His and the N-terminal region, respectively. It is therefore presumed that AII-1 and/or

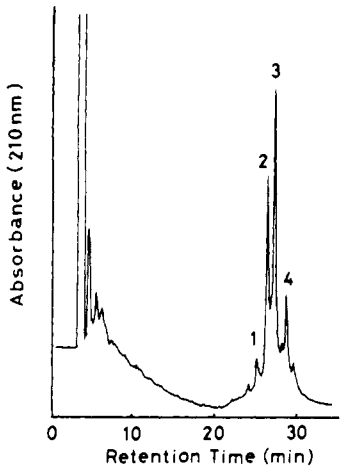

Figure 3. HPLC profile of the peptide treated with copper(II)/ascorbate. The reaction mixture (2 mL) in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.5 mg of peptide, 5 mM ascorbate, and 0.05 mM Cu(II) was incubated for 4 h at room temperature. Peaks 1, 2, 3, and 4 represent angiotensin II, AII-1, AII-2, and AII-3, respectively.

Table II. Amino Acid Composition of the Oxidized Peptides

amino acid	molar ratio,[a] %		
	AII-1	AII-2	AII-3
Asp	11.7	1.6	2.9
Arg	13.5	5.0	6.5
Val	12.4	5.3	4.0
Tyr	12.0	16.6	15.3
Ile	13.3	16.5	20.0
His	1.8	14.4	2.0
Pro	14.9	20.1	18.6
Phe	14.6	19.6	19.6

[a] Molar ratio (%) represents the mole concentration of each amino acid per total amino acids.

Table III. Automated Edman Degradation of AII-1

sequence cycle	PTH derivative[a]	yield, pmol	sequence cycle	PTH derivative[a]	yield, pmol
1	Asp	670.4	5	Ile	1936.4
2	Arg	187.8	6	X	377.3
3	Val	2204.5	7	Pro	530.7
4	Tyr	766.1	8	Phe	437.1

[a] PTH deivative of the amino acids represents the main compound obtained in each sequence cycle. X was detected as asparagine.

AII-2 can undergo further reaction by Cu(II)/ascorbate to generate AII-3.

To characterize the oxidized His in AII-1, the modified peptide was analyzed by automated Edman degradation, FAB-MS, and ^{1}H NMR spectrometry. In automated Edman degradation, the oxidized His (X) was detected as asparagine (Table III), whereas the FAB-MS spectrum of the modified peptides gave a signal corresponding to the $(M + H)^+$ ion, m/z 1063, suggesting mono-oxygenation of the substrate (Figure 4). In addition, the ^{1}H NMR spectrum of the modified peptides clearly revealed the alteration of the imidazole moiety (Figure 5). A new signal appeared at 6.18 ppm, which corresponds to an unconjugated olefin proton, suggesting the loss of the conjugated system of the imidazole ring. These spectral data of the modified peptides were quite analogous to those of the 2-imidazolone compounds obtained from histidine derivatives (Uchida and Kawakishi, 1986, 1989a, 1990c) and His-containing peptides (Uchida and Kawakishi, 1990a,b). As reported previously, the PTH derivative of 2-imidazolone amino acid can be eluted at the same position as that of PTH asparagine in the automated Edman degradation system (Uchida and Kawakishi, 1990b). Therefore, we consider that the oxidized His (X residue) within AII-1 should have a 2-imidazolone structure (Figure 6). The 2-imidazolone compound is the most characteristic product in the oxidation of histidyl compounds with Cu(II)/ascorbate, and we have recently proposed the oxocopper(III) species as the direct oxidizing agent for formation of the 2-imidazolone structure (Uchida and Kawakishi, 1990b,c).

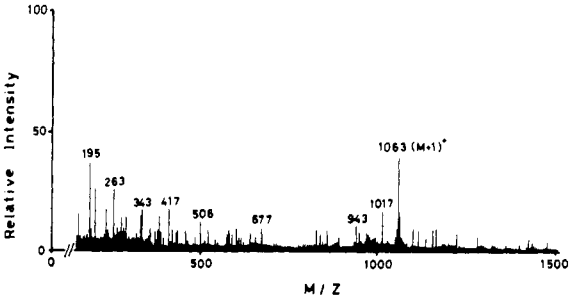

Figure 4. FAB-MS spectrum of AII-1.

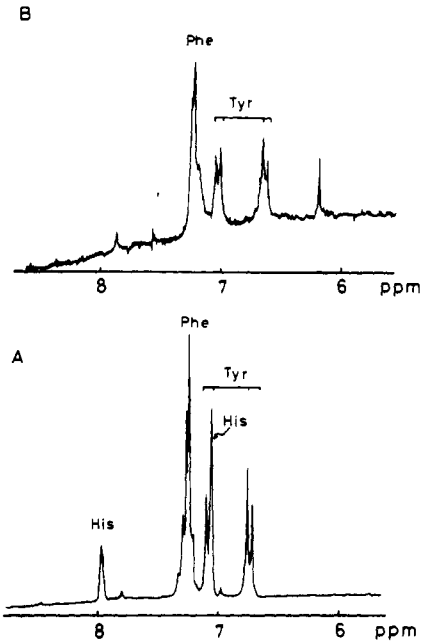

Figure 5. ^{1}H NMR spectrum of native peptide (A) and AII-1 (B).

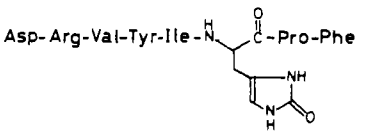

Figure 6. Sequence of AII-1.

On the other hand, neither AII-2 nor AII-3 gave any phenylthiohydantoin derivative of the N-terminal amino acid in automated Edman degradation (data not shown), suggesting the oxidation of the free amino group of N-terminal amino acid. So far, we have not obtained any information about their sequences from FAB-MS and ^{1}H NMR spectrometry. Hence, we next focused on the reactivity of N-terminal sequence of these peptides. To characterize the reactivity of the N-terminal region in detail, native peptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was digested with thermolysin to get several fragment peptides (Asp-Arg, Val-Tyr, Asp-Arg-Val-Tyr, and Ile-His-Pro-Phe). As shown in Table IV, it is of specific interest to note that the peptide Ile-His-Pro-Phe failed to react with Cu(II)/ascorbate, while the oxidation rate of other peptides is roughly proportional to the sequence. Reac-

Table IV. Rate of Oxidation of Thermolysin Digestion Products with Copper(II)/Ascorbate[a]

peptide	oxidation, μmol L^{-1} h^{-1}	peptide	oxidation, μmol L^{-1} h^{-1}
Val-Tyr	19.2	Asp-Arg-Val-Tyr	101.3
Arg-Val	35.0	Ile-His-Pro-Phe	0
Asp-Arg	62.3		

[a] The reaction mixture contained 0.5 mM peptide, 0.05 mM copper(II) ion, and 5 mM ascorbate in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4). The rate of oxidation (μmol L^{-1} h^{-1}) represents the rate of substrate (peptide) loss after 4 h of incubation.

tivities of preferred sequences were Asp-Arg-Val-Tyr > Asp-Arg > Arg-Val > Val-Tyr. It is thus definite that Asp-Arg-Val-Tyr is the sequence highly susceptible to oxidation by Cu(II)/ascorbate. In addition, these results suggest that the N-terminal sequence should have a key role in the oxidation of both N-terminal amino acids and His in the peptide. Although the reaction of Asp-Arg-Val-Tyr with Cu(II)/ascorbate gave several products (data not shown), we have not succeeded in the characterization of their chemical structures. Other than Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II), we have observed that an analogous peptide, Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin III), also exhibited such sequence-specific reactivity at the N-terminal region (Arg-Val-Tyr) with Cu(II)/ascorbate (data not shown), whereas the peptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (angiotensin I) was modified only at the histidine residues as reported previously (Uchida and Kawakishi, 1990b). These results suggest that the binding site of copper ion may affect the site of modification in peptides and proteins.

It is surprising that the peptide Ile-His-Pro-Phe corresponding to the C-terminal sequence of native peptide failed to react completely with Cu(II)/ascorbate (Table IV). Loss of the reactivity of Ile-His-Pro-Phe with Cu(II)/ascorbate may not be due to the inactivation of copper ions with chelation, since the peptide Gly-Gly-His which binds copper ions tightly undergoes modification with Cu(II)/ascorbate followed by selective loss of His within the peptide (Uchida and Kawakishi, 1989b). Therefore, it is obvious that the N-terminal sequence affects the reactivity of His with Cu(II)/ascorbate. At present, we theorize that the N-terminal region of the peptide is responsible for generation of active species which attack both the N-terminal region by itself and His; however, details remain to be further elucidated.

The site-specific oxidative modification of the peptide with Cu(II)/ascorbate could be of significance for the following reasons: (i) ascorbate, Cu(II), histidine, and its related materials are normal food and biological constituents; (ii) copper ions are present in a number of biological active proteins and can be bound to a large number of proteins in stoichiometric amounts, probably at a specific binding sites; and (iii) site-specific oxidation of the peptide happens at physiological pH, ionic strength, and temperature. Therefore, it is highly probable that protein undergoes oxidative modification by copper and reducing agents during food preservation and/or food processing. In addition, a huge number of materials may function as the reducing agent for copper ions in food. We have concluded that Amadori rearrangement products, which have been known as the main products in the initial step of amino-carbonyl reaction (Kawakishi et al., 1990; Cheng et al., 1991), and their degraded product, glucosone, can enhance the reduction of copper ion to oxidize histidine residues in protein (Kawakishi et al., 1991). Moreover, various reducing saccharides and metabolites such as dihydroxyacetone, glyceraldehyde, and even fructose catalyze the oxidative modification of protein (Kawakishi and Uchida, unpublished result). In cellular systems, it has been known that many enzymes are inactivated by metal-catalyzed oxidation systems and that this renders them susceptible to proteolytic degradation by a variety of exogenous and endogenous proteinases (Stadtman and Oliver, 1991). We trust that our present findings are significant to interpret chemically these site-specific mechanisms of protein oxidation induced by the metal-catalyzed systems.

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