# Ascorbate-Mediated Specific Modification of Histidine-Containing Peptides

Koji Uchida* and Shunro Kawakishi

In the presence of a catalytic amount of copper(II) ion, ascorbate stimulated the oxidative damage to the peptides via selective loss of histidine residues. The modified peptides yielded considerable amounts of ammonia and trace amounts of aspartate, glutamate, and serine with the acid treatment of the modified peptides. Such specific action of ascorbate toward the histidine residues gave rise to the depolymerization of polyhistidine accompanied by a significant loss of histidine residues. A $^{13}C$ NMR study clearly revealed the preferential binding of Cu(II) ion to the histidine residue in a peptide. It is therefore estimated that the selective reaction of ascorbate to the histidine residue should be attributed to the specific reaction of the histidine residue with copper ion.

Ascorbate is definitely an essential compound and is present at high concentrations in some mammalian tissues such as adrenals, leucocytes, brain, eyes, or pneumocytes (Lewin, 1976). As it is an important reductant in food and biological systems, ascorbate by itself has been the focus of numerous basic studies (Bates, 1981). 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 (Bielski, 1982).

Ascorbate is relatively stable in pure water, while in the presence of a catalytic amount of metal ion it is rapidly oxidized to dehydroascorbate through an electron transfer from ascorbate to metal (Khan et al., 1967a,b, 1968; Ogata et al., 1968). The rate of reaction is known to depend on pH, catalyst, oxygen pressure, temperature, etc. Especially, the acceleration of autoxidation of ascorbate by copper(II) ion is well-known, which is accompanied by the one-electron reduction of molecular oxygen to yield some free-radical species such as superoxide ($O_2^-$) and the hydroxyl radical ($^{\bullet}OH$) (Khan et al., 1967). The function of ascorbate is to reduce the metal ion [M($n$+1)] (eq 1) and to

$$M(n+1) + \text{ascorbate} \rightarrow M(n) + \text{dehydroascorbate} \quad (1)$$

$$M(n) + O_2 \rightarrow M(n+1) + O_2^- \quad (2)$$

$$O_2^- + 2H^+ \rightarrow H_2O_2 \quad (3)$$

$$M(n) + H_2O_2 \rightarrow M(n+1) + OH^- + {}^{\bullet}OH \quad (4)$$

serve as a source for $O_2^-$ (eq 2) and $H_2O_2$ (eq 3). The reduced metal ion [M($n$)] is conducted via a Fenton's reaction to generate the most potent oxidant, $^{\bullet}OH$ (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 (Samuni et al., 1983).

In vitro, a metal/ascorbate system promotes the oxidative scission of various food and biological materials such as polysaccharides (Uchida and Kawakishi, 1986a), proteins (Samuni et al., 1983; Shinar et al., 1983; Levine, 1984; Marx and Chevion, 1985; Uchida and Kawakishi, 1988), and DNA (Chiou, 1983, 1984). Especially on reaction with protein, this system gives rise to the specific modification of the histidine residues in the protein (Levine, 1984; Marx and Chevion, 1985; Uchida and Kawakishi, 1988). We have confirmed that approximately 60% of histidine residues in bovine serum albumin was selectively modified within 24 h through reaction with the Cu(II)/ascorbate system (Uchida and Kawakishi, 1988). On the basis of these observations, we have attempted to characterize the mechanism for specific modification of the histidine residue using a well-known copper-chelating peptide Gly-Gly-His and other His-containing peptides as the substrates.

In this study, we demonstrate the proposed mechanism for specific modification of the peptides by ascorbate, in which the reaction is triggered by the one-electron reduction of copper ion bound to the histidine residue followed by the formation of an active metal–peroxo complex.

## MATERIALS AND METHODS

**Materials.** L-Ascorbate was purchased from Wako Pure Chemical Industries Ltd. (Osaka). Glycylglycyl-L-histidine (Gly-Gly-His) was obtained from the Peptide Institute, Inc. (Osaka), and L-histidyl-L-phenylalanine (His-Phe), L-histidyl-L-tyrosine (His-Tyr), hippurylglycylglycine (Bz-Gly-Gly-Gly), and polyhistidine (poly-His) (MW $1.1 \times 10^4$) were from Sigma Chemical Co. Trifluoroacetic acid (TFA) (>99%) was purchased from Tokyo Kasei Kogyo Co., Ltd. Other reagents were of the highest grade commercially available.

**Reaction of the His-Containing Peptides with Ascorbate in the Presence of Copper(II) Ion.** The reaction was carried out at room temperature. The solution (25 mL) contained 0.08% poly-His or 1 mM peptide, 5 mM ascorbate, and 0.05 mM $CuSO_4$ in phosphate buffer (pH 7.2). The reaction was initiated by the addition of ascorbate and stopped by the addition of EDTA solution (0.04 mM).

**Copper(II)–Gly-Gly-His Complex.** The reaction of Gly-Gly-His with copper(II) ion was carried out in 5 mL of phosphate buffer (pH 7.2) containing 10 mM peptide and 0.5 mM $CuSO_4$. Changes in the visible absorption of the Cu(II)–peptide complex induced by the addition of 50 mM ascorbate were measured with a Hitachi Model 200-10 spectrophotometer.

**High-Performance Liquid Chromatography (HPLC).** Chromatographic determination of poly-His was performed by HPLC on a TSK-GEL G3000 PW column (7.5 × 600 mm). Since the reaction mixture was emulsified, 0.5 mL of HCl (0.2 N) was added to a portion of the mixture (1.0 mL) each time to dissolve poly-His and then subjected to HPLC analysis. Samples were eluted at a rate of 1.0 mL/min with 0.1 M phosphate buffer (pH 4.4) containing 0.1 M KCl, the elution being monitored at 210 nm.

Chromatographic determination of other smaller peptides, Gly-Gly-His, His-Phe, His-Tyr, and Bz-Gly-Gly-Gly, was also done by HPLC on a Develosil ODS-5 (5 × 250 mm) column. Chromatographic conditions were as follows: samples were eluted at a rate of 0.8 mL/min with a TFA solution (0.1%) for Gly-Gly-His, TFA (0.1%)–methanol (3:1) for His-Phe and Bz-Gly-Gly-Gly, and TFA (0.1%)–methanol (5:1) for His-Tyr, the elutions being monitored at 210 nm.

Areas of the chromatographic peaks of each substrates were calculated by use of a Shimadzu Chromatopac integrator, Model C-R3A.

**Amino Acid Composition.** Amino acid analysis was performed with a JEOL JLC-6AH amino acid analyzer for which the

Department of Food Science and Technology, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-01, Japan.

0021-8561/89/1437-0897$01.50/0 

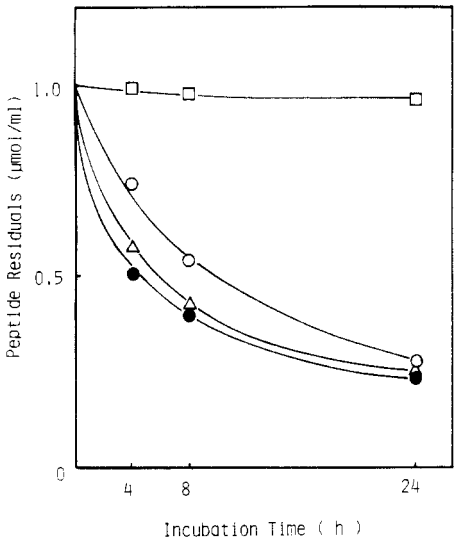


**Figure 1.** Time-dependent loss of the peptides during incubation with the Cu(II)/ascorbate system. Symbols: ○, Gly-Gly-His; △, His-Tyr, ●, His-Phe; □, Bz-Gly-Gly-Gly.

**Table I. Time-Dependent Changes in the Amino Acid Composition of Glycylglycyl-L-histidine through Reaction with the Cu(II)/Ascorbate System**

| | molar ratio,[a] % | | | |
|---|---|---|---|---|
| | 0 h | 4 h | 8 h | 24 h |
| Asp | 0 | 1.6 | 2.5 | 4.1 |
| Ser | 0 | 0 | 0.4 | 0.7 |
| Glu | 0 | 0 | 0.4 | 0.6 |
| Gly | 65.0 | 63.4 | 61.5 | 61.0 |
| His | 33.3 | 28.5 | 27.0 | 13.8 |
| $NH_4OH$ | 1.7 | 6.5 | 8.2 | 20.3 |

[a] Molar ratio was represented by the mole concentration of each amino acid per total amino acids.

sample was prepared as follows: The mixtures at each time were freeze-dried and then hydrolyzed with 6 N HCl at a concentration of 2 mg of peptide/mL at 120 °C for 24 h. The hydrolysates were concentrated, dissolved in aqueous HCl (pH 2.2), and then submitted to the amio acid analysis.

**$^{13}C$ NMR Spectrometry.** Gly-Gly-His (20 mg) was dissolved in 5 mM ascorbate or 0.1 mM $CuSO_4$ in a phosphate buffer (25 mL, pH 7.2), and after the mixtures were freeze-dried, they were dissolved in 0.6 mL of $D_2O$ and then submitted to the measurement using 1,4-dioxane as an internal standard. Proton noise decoupled $^{13}C$ NMR spectra of the sample solutions in 5-mm (i.d.) tubes were recorded by a JEOL JMN-FX 200 (50.2-MHz) spectrometer.

## RESULTS AND DISCUSSION

**Selective Damage to the Histidine Residues in the Peptides.** Figure 1 shows the time-dependent losses of the pepetides during incubation with ascorbate in the presence of copper(II) ion. The Cu(II)/ascorbate system was highly reactive to the His-containing peptides (Gly-Gly-His, His-Tyr, His-Phe), and approximately 70% of each peptide was diminished within 24 h of incubation. The non-histidyl peptide, Bz-Gly-Gly-Gly, remained intact. In regard to this, other non-histidyl materials such as cysteine, lysine, arginine, tyrosine, phenylalanine, glutathione, and their derivatives were all poor in reactivity with the Cu(II)/ascorbate system (data not shown). Loss of the His-containing peptides in the Cu(II)/ascorbate system was greatly accelerated in an $O_2$ atmosphere while entirely retarded in a $N_2$ atmosphere (Uchida et al., unpublished data). The reaction was therefore confirmed to be $O_2$ dependent, which strongly suggests the participiation of oxygen-derived free radicals.

**Table II. Time-Dependent Changes in the Amino Acid Composition of Histidylphenylalanine through Reaction with the Cu(II)/Ascorbate System**

| | molar ratio,[a] % | | | |
|---|---|---|---|---|
| | 0 h | 4 h | 8 h | 24 h |
| Asp | 0 | 0.1 | 0.7 | 1.2 |
| Ser | 0 | 0 | 0 | 0.7 |
| Glu | 0 | 0 | 0.1 | 0.7 |
| Phe | 49.4 | 50.0 | 50.7 | 50.8 |
| His | 49.4 | 38.9 | 33.3 | 23.1 |
| $NH_4OH$ | 1.2 | 11.1 | 14.5 | 23.1 |

[a] Molar ratio was represented by the mole concentration of each amino acid per total amino acids.

**Table III. Time-Dependent Changes in the Amino Acid Composition of Histidyltyrosine through Reaction with the Cu(II)/Ascorbate System**

| | molar ratio,[a] % | | | |
|---|---|---|---|---|
| | 0 h | 4 h | 8 h | 24 h |
| Asp | 0 | 0.3 | 0.8 | 1.3 |
| Ser | 0 | 0 | 0.1 | 0.8 |
| Glu | 0 | 0 | 0.1 | 0.8 |
| Tyr | 50.0 | 51.6 | 50.0 | 50.0 |
| His | 48.8 | 40.6 | 33.3 | 24.2 |
| $NH_4OH$ | 1.2 | 7.5 | 15.2 | 22.6 |

[a] Molar ratio was represented by the mole concentration of each amino acid per total amino acids.

**Table IV. Comparison of the Amino Acid Composition between Native and Modified Poly-L-histidine**

| | molar ratio,[a] % | | |
|---|---|---|---|
| | native | modified (24 h) | difference |
| Asp | 0 | 2.9 | +2.9 |
| Ser | 0 | 3.7 | +3.7 |
| Glu | 0 | 4.4 | +4.4 |
| His | 96.2 | 73.1 | -23.1 |
| $NH_4OH$ | 3.8 | 15.9 | +12.1 |

[a] Molar ratio was represented by the mole concentration of each amino acid per total amino acids.

Then, we characterized the change in amino acid composition of the peptides (Tables I–III). In the case of Gly-Gly-His, the histidine residue in the peptide was selectively damaged while the glycine residues remained almost intact (Table I). Therefore, it seems little doubt that the decrease of the peptide was predominantly due to the loss of histidine residue. Accompanying the selective loss of histidine residue, a considerable amount of ammonia was produced by the acid treatment of the modified peptide and, moreover, trace amounts of aspartate, glutamate, and serine were also detected. We believe that the selective damage to the histidine residue in the peptide might have led to the liberation of ammonia from the oxidized imidazole moiety followed by the formation of acidic amino acids.

This result was entirely analogous to those of other His-containing peptides (Tables II and III).

**Depolymerization of Polyhistidine.** Synthetic poly(amino acids) like poly-His can often be used as instructive protein models, and their combination with metal ions exhibits some resemblance to metalloproteins. Actually, poly-His shows high affinity with copper ions, which exhibits the ascorbate oxidase like activity (Pecht and Anbar, 1965; Pecht et al., 1967). Hence, we determined the change of the homopolymer of histidine through reaction with ascorbate in the presence of copper(II) ion.

Figure 2 demonstrates the HPLC profile on a TSK-GEL G3000 PW column, in which the formation of smaller peptides is accompanied by the decrease of native poly-His.

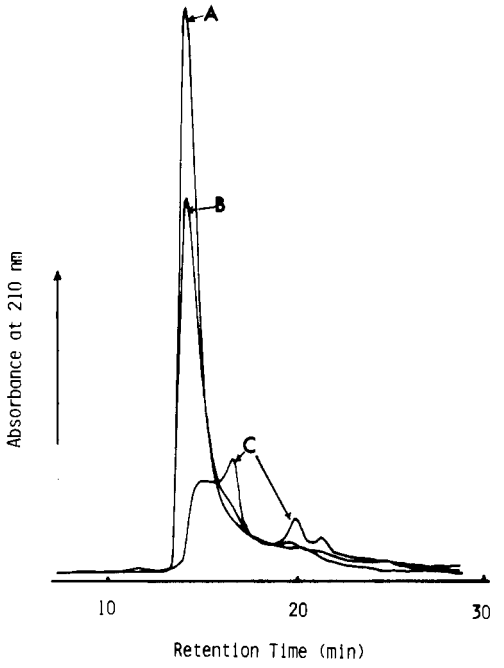


**Figure 2.** Time-dependent changes in the HPLC chromatogram of polyhistidine during incubation with the Cu(II)/ascorbate system: (A) control polyhistidine; (B) 8 h; (C) 24 h.

It was therefore confirmed that the reaction of histidine residues with the Cu(II)/ascorbate system could result in the cleavage of the peptide itself. Then, we characterized the change in amino acid composition of the peptide (Table IV). Similar to the above data (Tables I–III), the hydrolysate of modified poly-His produced a considerable amount of ammonia and trace amounts of aspartate, serine, and glutamate accompanied by the loss of histidine residue.

In connection with the free-radical modification of poly-His, Cooper et al. (1985) have reported that, by using hydrogen peroxide ($H_2O_2$) instead of ascorbate as a catalyst, most of the histidine residues in poly-His could be converted into aspartic acid (approximately 80%) by an acid treatment of modified poly-His. However, we could not have confirmed such a drastic conversion of poly-His through the reaction with ascorbate. This suggests the difference of the active species and/or mechanism in both systems.

Consequently, these accumulated data suggested that, in the presence of copper(II) ion, ascorbate preceded the cleavage of the peptide accompanied by the selective modification of histidine residues. In addition, as presented in eq 5, these specific damages to the histidine residues resulted secondarily in the formation of ammonia and acidic amino acids with the acid hydrolysis of the modified histidine residue (X) in the peptides.

$$\text{His} \xrightarrow{\text{Cu(II)/ASA}} [\text{X}] \xrightarrow{\text{hydrolysis}} \text{NH}_3, \text{Asp, Glu, Ser} \quad (5)$$

**Copper–Imidazole Complex.** Considering the amino acid sequences and the nature of the copper-binding site of albumins, Gly-Gly-His has been chosen to mimic the native Cu(II) transport site of albumin (Sarker and Wigfield, 1968; Lau et al., 1974; Kruck and Sarkar, 1975). This peptide has the same ligand atoms binding to copper(II) ion as those suggested for albumin. Hence, we attempted to interpret the mechanism for the selective modification of histidine residue through the specific copper-binding ability of the imidazole group.

The reaction of Gly-Gly-His with copper(II) ion produced a light red 1:1 complex, which exhibits an absorption

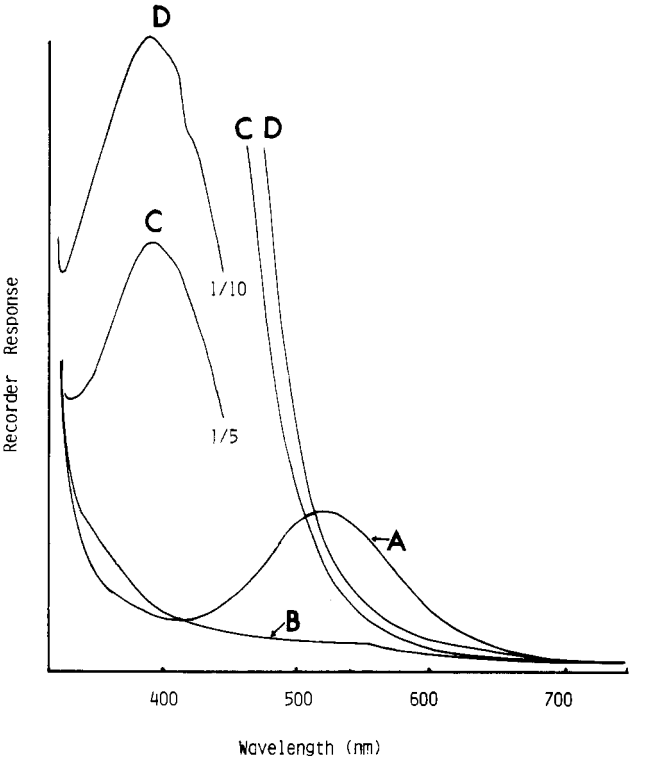


**Figure 3.** Changes in the visible absorption of the Cu(II)–glycylglycylhistidine complex by the addition of ascorbate. Each spectrum represents the reaction mixtures of Cu(II)–peptide (A) and Cu(II)–peptide with ascorbate after 1 min (B), 60 min (C), and 180 min (D).

maximum at 525 nm (spectrum A in Figure 3). This clearly indicates the specific reaction of this peptide with copper(II) ion (Sarker and Wigfield, 1968; Lau et al., 1974). In addition, we confirmed the specific affinity of the histidyl moiety with copper(II) ion in this complex by $^{13}C$ NMR spectrometry (Figure 4). In the presence of copper(II) ion (spectrum C in Figure 4), the signals derived from the imidazole moiety (134.6, 131.4, 117.4, and 28.5 ppm, respectively) were significantly broadened, while such specific broadenings were not observed in the presence of ascorbate (spectrum B in Figure 4). The result strongly suggests the existence of a specific interaction between the imidazole group and copper ion. The reaction of histidine with copper(II) ion could possibly be accompanied by a charge transfer from the histidine ring orbital to copper ions (Sarker and Wigfield, 1968). It can therefore be presumed that the change in signals is due to the formation of an electron-transfer complex between the imidazole side chain and copper ions as follows:

$$[\text{His}\cdots\text{Cu(II)}] \leftrightarrow [\text{His}^{+}\cdots\text{Cu(I)}] \quad (6)$$

The characteristic absorption of this copper–peptide complex was completely diminished by the addition of ascorbate (spectrum B in Figure 3). This implies the likelihood of the one-electron reduction of the His–Cu(II) complex to the colorless His–Cu(I) complex by ascorbate (Kimura et at., 1986). Subsequently, the mixture assumed gradually a light yellow color, and the new absorption band, which might be due to the accumulation of oxidized products of Gly-Gly-His, appeared near 400 nm (spectra C and D in Figure 3).

**Reaction Mechanism.** The next step appears to involve a multistep free-radical or non-free-radical route, and the mechanism of each step is by no means clear. Nevertheless, we speculate a reaction scheme with an electron transfer to molecular oxygen.

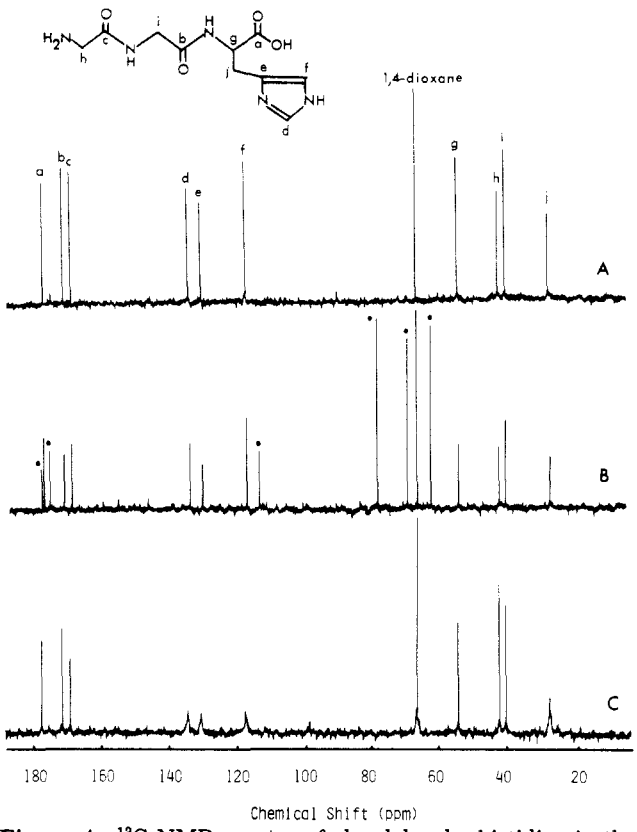


**Figure 4.** $^{13}C$ NMR spectra of glycylglycyl-L-histidine in the presence of ascorbate or Cu(II) ion: (A) Gly-Gly-His; (B) Gly-Gly-His + ascorbate; (C) Gly-Gly-His + Cu(II). In spectrum B, the ● indicates the signals derived from ascorbate.

As mentioned above, the process may definitely involve an electron transfer from ascorbate to copper(II) ion bound to the histidine residue (eq 7). The Cu(I)–His complex

$$\text{Cu(II)–His} \xrightarrow{\text{ascorbate}} \text{Cu(I)–His} \quad (7)$$

$$\text{Cu(I)–His} + O_2 \rightarrow O_2\text{–Cu(I)–His} \quad (8)$$

$$O_2\text{–Cu(I)–His} \rightarrow O_2^-\text{–Cu(II)–His} \quad (9)$$

reacts subsequently with molecular oxygen to give an $O_2$ adduct (eq 8). It is probable that the His–Cu(I)–$O_2$ complex is followed by generation of the His–Cu(II)–$O_2^-$ complex (eq 9). At present, we assume that the metal–peroxo complex like $O_2$–Cu(I) or $Co_2^-$–Cu(II) can react directly with the ligand (histidine residue) itself. But we do not have any direct evidence to support our hypothesis.

As to the participation of the hydroxyl radical (•OH), we speculate that a diffusible •OH cannot cause such selective damage toward histidine residues alone. Actually, the loss of histidine residues in the protein through the incubation with the Cu(II)/ascorbate system could not be prevented by the scavengers of •OH (Uchida and Kawakishi, 1988).

We have not yet characterized the oxidized form of Gly-Gly-His and other peptides. However, similar types of oxygenated or ring-ruptured products of histidine residue, as those confirmed through the reaction of *N*-benzoylhistidine with $^1O_2$ (Tomita et al., 1969) or the copper/ascorbate system (Uchida and Kawakishi, 1986b), might be formed.

We presented, herein, the proposed mechanism concerning a series of electron transfers from ascorbate to the copper–histidine complex. Our present findings may have significance to interpret the site-specific mechanism for the protein modification with ascorbate and to exploit a new function of ascorbate in free-radical chemistry. We are now planning to detect unstable reaction intermediates of the imidazole ring to elucidate the total oxidation route of histidine residue.

We believe that the specific reaction in this study may actually result in the denaturation or inactivation of protein in food and biological systems. Such reactions must be physiologically important in connection with the oxygen toxicity or cellular proteolysis on the mixed-function oxidation systems. From a nutritional aspect, specific damage to the histidine residues in protein may result in the histidine deficiency or in lowering the nutritive value of foods.

These findings in the selective reaction toward the His-containing peptides are expected to be followed by the chemical and biological evaluation of damaged protein in food and biological systems.

**Registry No.** Cu, 7440-50-8; L-His, 71-00-1; Gly-Gly-His, 7451-76-5; His-Phe, 16874-81-0; His-Tyr, 35979-00-1; poly-His, 26062-48-6; poly-His,SRU, 26854-81-9; ascorbate, 50-81-7.

## LITERATURE CITED

Bates, C. J. The Function and Metabolism of Vitamin C in Man. In *Vitamin C (Ascorbic Acid)*; Counsell, J. N., Hornig, D. H., Eds.; Applied Science: London, 1981; p 1.

Bielski, B. H. J. Chemistry of Ascorbic Acid Radicals. In *Ascorbic Acid: Chemistry, Metabolism, and Uses*; Seib, P. A., Tolbert, B. M., Eds.; American Chemical Society: Washington, DC, 1982; p 81.

Chiou, S. H. DNA- and Protein-Scission Activities of Ascorbate in the Presence of Copper Ion and a Copper-Peptide Complex. *J. Biochem.* **1983**, *94*, 1259–1267.

Chiou, S. H. DNA-Scission Activities of Ascorbate in the Presence of Metal Chelates. *J. Biochem.* **1984**, *96*, 1307–1310.

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

Khan, M. M. T.; Martell, A. E. Metal Ion and Metal Chelate Catalyzed Oxidation of Ascorbic Acid by Molecular Oxygen. I. Cupric and Ferric Ion Catalyzed Oxidation. *J. Am. Chem. Soc.* **1967a**, *89*, 4176–4185.

Khan, M. M. T.; Martell, A. E. Metal Ion and Metal Chelate Catalyzed Oxidation of Ascorbic Acid by Molecular Oxygen. II. Cupric and Ferric Chelate Catalyzed Oxidation. *J. Am. Chem. Soc.* **1967b**, *89*, 7104–7111.

Khan, M. M. T.; Martell, A. E. Kinetics of Metal Ion and Metal Chelate Catalyzed Oxidation of Ascorbic Acid. III. Vanadyl Ion Catalyzed Oxidation. *J. Am. Chem. Soc.* **1968**, *90*, 6011–6017.

Kimura, E.; Koike, T.; Shimizu, Y.; Kodama, M. Complexes of the Histamin $H_2$-Antagonis Cimetidine with Divalent and Monovalent Copper Ions. *Inorg. Chem.* **1986**, *25*, 2242–2246.

Kruck, T. P. A.; Sarkar, B. Equilibria and Structures of the Species in the Ternary System of L-Histidine. Copper(II), and Diglycyl-L-histidine, a Peptide Mimicking the Copper(II)-Transport Site of Human Serum Albumin. *Inorg. Chem.* **1975**, *14*, 2383–2388.

Lau, S.; Kruck, T. P. A.; Sarkar, B. A Peptide Molecule Mimicking the Copper(II) Transport Site of Human Serum Albumin. *J. Biol. Chem.* **1974**, *249*, 5878–5884.

Levine, R. L. Mixed-Function Oxidation of Histidine Residues. *Methods Enzymol.* **1984**, *107*, 370–377.

Lewin, S. Biological Activity and Potential. In *Vitamin C: Its Moleullar Biology and Medical Potential*; Lewin, S., Ed.; Academic: London, 1976; p 75.

Marx, G.; Chevion, M. Site-Specific Modification of Albumin by Free Radicals. *Biochem. J.* **1985**, *236*, 397–400.

Ogata, Y.; Kosugi, Y.; Morimoto, T. Kinetics of the Cupric Salt-Catalyzed Autoxidation of L-Ascorbic Acid in Aqueous Solutions. *Tetrahedron* **1968**, *24*, 4057–4066.

Pecht, I.; Anbar, M. Oxidase-like Activity of the Copper(II) Poly-L-histidine Complex. *Nature* **1965**, *207*, 1386–1387.

Pecht, I.; Levitzki, A.; Anbar, M. The Copper-Poly-L-histidine Complex. I. The Environmental Effect of the Polyelectrolyte

on the Oxidase Activity of Copper Ions. *J. Am. Chem. Soc.* **1967**, *89*, 1587–1591.
Samuni, A.; Aronovitch, J.; Godinger, D.; Chevion, M.; Czapski, G. On the Cytotoxicity of Vitamin C and Metal Ions. *Eur. J. Biochem.* **1983**, *137*, 119–124.
Sarker, B.; Wigfield, Y. Evidence for Albumin-Cu(II)-Amino Acid Ternary Complex. *Can. J. Biochem.* **1968**, *46*, 601–607.
Shinar, E.; Navok, T.; Chevion, M. The Analogous Mechanisms of Enzymatic Inactivation Induced by Ascorbate and Superoxide in the Presence of Copper. *J. Biol. Chem.* **1983**, *258*, 14778–14783.
Tomita, M.; Irie, M.; Ukita, T. Sensitized Photooxidation of Histidine and Its Derivatives. Products and Mechanism of the Reaction. *Biochemistry* **1969**, *8*, 5149–5160.
Uchida, K.; Kawakishi, S. Oxidative Depolymerization of Polysaccharides Induced by the Ascorbic Acid-Copper Ion Systems. *Agric. Biol. Chem.* **1986a**, *50*, 2579–2583.
Uchida, K.; Kawakishi, S. Selective Oxidation of Imidazole Ring in Histidine Residues by the Ascorbic Acid-Copper Ion System. *Biochem. Biophys. Res. Commun.* **1986b**, *138*, 659–665.
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*, 1529–1535.

Received for review August 1, 1988. Accepted February 28, 1989.

# Polyphenoloxidase in Wild Rice (*Zizania palustris*)

Yaw J. Owusu-Ansah

Polyphenoloxidase (PPO) was isolated from wild rice (*Zizania palustris*). Partial purification by acetone precipitation and dialysis of the crude extract yielded a 2.8-fold purification of the activity of the extract. The optimum pH and temperature of the enzyme were 7.8 and 25 °C, respectively. The enzyme catalyzed the oxidation of *o*-diphenols but not monophenols. Thermal inactivation data indicated first-order kinetics with an apparent activation energy of 20 kJ/mol. Polyacrylamide gel electrophoresis showed the PPO to be isoenzymes with molecular weights of 116 000, 48 000, 42 000, and 35 000, respectively.

Polyphenoloxidase (PPO; *o*-diphenol:$O_2$ oxidoreductase, E.C. 1.14.18.1) is one of the enzymes involved in the oxidation of phenolic compounds to brown pigments. The enzyme has been extensively studied because of its relative ubiquity and importance in the food and agricultural industry. It has been found in green olives (Sciancalepore and Longone, 1984), bananas (Palmer, 1963; Galeazzi et al., 1981), avocados (Kahn, 1977), peaches (Jen and Kahler, 1974; Flurkey and Jen, 1978), mushrooms (Jolley et al., 1969; Yamaguchi et al., 1970), potatoes (Hyodo and Uritani, 1966; Weaver et al., 1968; Mondy and Koch, 1978; Batistuti and Lourenço, 1985), and artichokes (Zawistowski et al., 1986). In all these commodities, the enzyme is implicated in the undesirable browning of the products, which ultimately reduces their marketability. Except for tea fermentation, the browning reaction catalyzed by PPO has not been positively associated with food quality.

Wild rice is a cereal of the *Zizania* family growing in the shallow and sluggish waters of Northern United States and Canada. The exotic crop, which commands premium price, requires unique processing protocol compared to other cereals. After harvest, the rice is cured, a process that involves biochemical transformation of the rice grain to give its characteristic color and aroma. Qualitative presumptive tests (Withycombe, 1974) suggest that the curing process involves polyphenolase-mediated reactions in which inherent free phenolic acids in the rice are oxidized into brown and black pigments responsible for the color of the rice. The presence of polyphenolase and the characteristics of the enzyme in wild rice have not been clearly demonstrated. This study was undertaken to confirm the presence of polyphenolase activity in wild rice and to characterize the enzyme.

POS Pilot Plant Corporation, 118 Veterinary Road, Saskatoon, Saskatchewan S7N 2R4, Canada.

## MATERIALS AND METHODS

**Materials.** Freshly harvested wild rice was obtained from the La Ronge Wild Rice processing plant. The rice was separated into maturity groups at the La Ronge plant by a combination of the following tests: visual color examination, moisture content, and bulk density. The fully matured rice, with a moisture content range of 25–30%, a bulk density range of 0.40–0.50 g/cm$^3$, and olive green to brown color, was shipped on solid carbon dioxide in a cooler and was kept frozen until used.

**Methods.** *Enzyme Extraction.* This was performed essentially as described by Flurkey and Jen (1978). The wild rice samples were thawed, and 100-g samples were homogenized for 2 min in a Waring blender with 200 mL of cold acetone (−20 °C) and 5.0 g of poly(ethylene glycol) as a phenolic scavenger. The slurry was suction-filtered and the pellet reextracted with cold acetone (−20 °C). The pellet was quantitatively transferred to a 500-mL beaker and homogenized for 2 min on a Polythron homogenizer with 200 mL of cold acetone. The slurry was suction-filtered and washed with another 200 mL of cold acetone. The powder was air-dried overnight at room temperature in a fumehood to remove residual acetone. The acetone powder was kept frozen at −20 °C until use. A 5-g portion of the acetone powder thus obtained was suspended in 200 mL of 0.05 M potassium phosphate buffer, pH 6.2, containing 1 M KCl. The suspension was stirred for 30 min at 4 °C and then centrifuged at 12000*g* for 20 min at 4 °C. The supernate was carefully decanted and was considered the "crude enzyme extract".

*Partial Purification Procedure.* The partial purification was performed essentially as described by Galeazzi et al. (1981). Two volumes of cold acetone (−20 °C) were slowly added to 50-mL aliquots of the crude enzyme extracts, and the resulting precipitate was separated by centrifugation at 12000*g* and 4 °C for 15 min. The precipitate was resolubilized with 0.2 M sodium phosphate buffer, pH 7.0, containing 0.25% Triton X-100 and recentrifuged. The supernate was dialyzed against 0.05 M phosphate buffer, pH

0021-8561/89/1437-0901$01.50/0 