Selective Oxidation of Tryptophan and Histidine Residues in Protein through the Copper-catalyzed Autoxidation of L-Ascorbic Acid

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The oxidative modification of bovine serum albumin with an ascorbic acid-copper ion system was studied. Under physiological conditions (pH 7.2, ambient temperature), this system mainly caused the modification of amino acid residues in the protein, and its polymerization was scarcely observed. The results of spectrophotometric assays and amino and analysis of the protein clearly suggested the selective damage to tryptophan and histidine residues. The reaction could be retarded by catalase and Cu(II)-chelating agents, while superoxide dismutase and hydroxyl radical scavengers showed little effect. These specific reactions were explained by the site-specific formation of the oxygen-derived free radical followed by its reaction with a specific site of the protein.

Oxygen radicals have been implicated by many authors to be important causative agents of the oxygen toxicity in cancer, aging and other human diseases. The generation of oxygen radicals including the superoxide (O₂⁻) and hydroxyl radical (·OH) during normal cellular metabolism has been suggested to be responsible for the action of various biological and synthetic materials as enzymes, antibiotics, carcinogen, and reducing materials under physiological conditions.

As to oxygen toxicity in biological systems, O₂⁻ has been regarded as the agent responsible for deleterious effects, however, it has also been found to exhibit low reactivity toward biological materials, which suggested that O₂⁻ by itself is not a deleterious agent but rather that it plays an important role as a source for highly reactive oxygen radicals. In aqueous media, O₂⁻ is immediately reduced to hydrogen peroxide (H₂O₂) via the dismutation reaction (reaction 1) and, subsequently, H₂O₂ is catalytically decomposed by a reduced metal ion to the hydroxyl radical (reactions 2 and 3).

\[ 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \]  \hspace{1cm} (1)

\[ \text{M}^{n+} + \text{O}_2^- \rightarrow \text{M}^{(n-1)+} + \text{O}_2 \]  \hspace{1cm} (2)

\[ \text{M}^{(n-1)+} + \text{H}_2\text{O}_2 \rightarrow \text{M}^{n+} + \text{OH}^- + \cdot \text{OH} \]  \hspace{1cm} (3)

The Hydroxyl radical, formed via reactions 2 and 3 (metal-catalyzed Haber–Weiss reaction), is the most powerful oxidizing species among several oxygen radicals and is able to oxidize most organic compounds via hydroxylation and hydrogen abstraction reactions. Although the reactivity of ·OH has been considered to be rapid and non-specific toward various compounds, a site-specific mechanism for ·OH as to protein damage has been suggested, in which metal ions are bound to proteins. Nevertheless, the detailed mechanism of this reactivity of ·OH toward protein has not been elucidated yet.

In this study, we chose the ascorbic acid model systems from a wide variety of generating systems for oxygen radicals to characterize the above specific reaction with protein. Ascorbic acid is an essential compound in human tissues and has been the focus of numerous basic scientific studies in relation to the enzymatic or non-enzymatic oxidation reactions in food and biological systems. In the presence...
of trace metal ions, ascorbic acid reacts with molecular oxygen and, during its autoxidation, \( \text{O}_2^- \) and hydrogen peroxide are formed.\(^8\)\(^{-11}\)

In the present study, concerning the modification of bovine serum albumin by a Cu(II)-catalyzed autoxidation system for ascorbic acid, we demonstrate the chemical alteration of the protein and the selective actions of free radicals on tryptophan and histidine residues.

**MATERIALS AND METHODS**

**Materials.** Bovine serum albumin and l-ascorbic acid were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo) and Wako Pure Chemical Industries Ltd. (Osaka), respectively. Other reagents were of the highest grade commercially available.

**Oxidation of bovine serum albumin.** The reactions were carried out at room temperature, the solutions (25 ml) containing 0.04% (w/v) bovine serum albumin, 2 mM ascorbic acid and 0.01 mM Cu(II) in phosphate buffer (pH 7.2). The reactions were initiated by the addition of ascorbic acid and stopped by the addition of an EDTA solution (0.04 mM).

Preparation of the samples for the measurement of native fluorescence, UV absorption and CD, and for the analysis of the amino acid composition was performed as follows. The reaction conditions were the same as those above, and, after terminating the reactions, the mixtures were freeze-dried and then desalted on a Sephadex G-25 column. Subsequently, the protein-containing fractions were collected, freeze-dried and then submitted to each analysis.

**High performance liquid chromatography (HPLC).** Chromatographic determinations of the protein was performed by HPLC on a TSK-GEL G3000 SW column (7.5 × 600 mm). Samples were eluted at a rate of 1.0 ml/min with 0.1 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, the elution being monitored continuously at 210 nm.

**Polyacrylamide gel electrophoresis.** Slab gels, 7.5% acrylamide, and electrophoresis buffer, Tris–glycine, were prepared as described by Davis.\(^{12}\) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% acrylamide) was performed according to Laemmli.\(^{13}\) Gel sheets were stained with a solution of 0.25% Coomassie Brilliant Blue R-250 in water–2-propanol–acetic acid (5:5:1, v/v/v) and destained with 7% acetic acid containing 5% methanol.

**Native fluorescence of the protein.** Native fluorescence of the protein was measured, with excitation at 280 nm and emission at 340 nm, with a JASCO FP-550A spectrophotometer according to the method of Gutteridge and Wilkins.\(^{51}\)

**CD spectra measurements.** CD spectra of samples (1 mg protein/ml) were measured at 25 °C with a JASCO J-40CS spectropolarimeter, mean residue ellipticities \([\theta]\) being calculated on the basis of average residue weights.

**Amino acid composition.** Amino acid analysis was performed with a JEOI JLC-6AH amino acid analyzer, for which the samples were prepared as follows. The reaction mixtures contained 0.04% bovine serum albumin, 2 mM ascorbic acid and 0.01 mM Cu(II) in phosphate buffer (25 ml, pH 7.2). After freeze-drying of the mixtures, they were desalted on Sephadex G-25 and then hydrolyzed with 6 N HCl at a concentration of 2 mg protein/ml at 120 °C for 24 h. The hydrolyzates were concentrated, dissolved in aqueous HCl (pH 2.2) and then submitted to the analysis.

**Tryptophan content.** For determination of the tryptophan content of the protein, alkaline-hydrolysis was carried out with 4 M sodium hydroxide at 120 °C for 24 hr according to the method of Hugi and Moore.\(^{14}\) The hydrolyzates were submitted to HPLC analysis on a reversed-phase column (Develosil ODS-5). Samples were eluted at a rate of 0.8 ml/min with 0.1% trifluoroacetic acid–methanol (3:1), the elution being monitored continuously at 280 nm.

**RESULTS**

**Oxidative modification of bovine serum albumin.** Reaction of the protein with the ascorbic acid–Cu(II) system was examined by HPLC on a TSK-GEL G3000 SW column and by polyacrylamide gel electrophoresis. Figure 1 shows the HPLC profiles of the native and oxidized proteins, the chromatographic peaks of the native (peak A) and polymerized (peak B) proteins were found to be lowered and broadened, and an increase in the lower molecular weight form of the protein (peak C) was observed, which was proportional to the incubation time. The pronounced tailing of the chromatographic peak of the native protein reflects the alteration of the protein on ascorbic acid oxidation followed by the increased affinity with the stationary phase. Furthermore, we characterized the alteration of the protein by SDS-polyacrylamide gel electrophoresis (Fig. 2). Upon incubation with as-
Oxidation of Protein with an Ascorbic Acid-Cu(II) system

Fig. 1. Time Dependent Changes in the HPLC Profiles of Oxidized Bovine Serum Albumin.

A reaction mixture containing 0.04% protein, 2 mM ascorbic acid and 0.01 mM Cu(II) in phosphate buffer (pH 7.2) was injected in each case (0, 4, 8 and 24 hr). Chromatographic peaks A, B and C correspond to the native protein, polymerized protein and oxidized products, respectively. Ascorbic acid and its degraded products were not eluted under these chromatographic conditions.

corobic acid and Cu(II), the major bands of the native protein and the minor bands of its polymers gradually disappeared and, within 8 hr incubation, more lower molecular weight forms, of between 28 kDa and 67 kDa, appeared. With additional incubation (24 hr), the oxidized products were further degraded to lower molecular weight forms, of below 14 kDa. High molecular weight forms of the protein were not observed, and oxidative de-polymerization of the protein was presumed to be the predominant reaction in this system. The native and oxidized forms of the protein were further examined by native-polyacrylamide gel electrophoresis (Fig. 3). After 24 hr incubation, the native protein band had almost completely disappeared, and a great extent of

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Protein Exposed to Ascorbic Acid in the Presence (B) and Absence (A) of Cu(II).

The reaction mixtures contained 0.04% bovine serum albumin, 2 mM ascorbic acid and 0.01 mM Cu(II) in phosphate buffer (25 ml, pH 7.2), and 0.5 ml portions of the mixtures were applied each time (0, 4, 8 and 24 hr). The protein was precipitated by the addition of 0.5 ml of a trichloroacetic acid solution (12%), at 4°C for 24 hr and then submitted to analysis.

Fig. 3. Native-polyacrylamide Gel Electrophoresis of the Protein Exposed to Ascorbic Acid in the Presence (B) and Absence (A) of Cu(II).

The reaction mixtures contained 0.04% bovine serum albumin, 2 mM ascorbic acid and 0.01 mM Cu(II) in phosphate buffer (25 ml, pH 7.2), and 0.15 ml portions of the mixtures were applied each time (0, 4, 8 and 24 hr) and, after the addition of 0.05 ml of glycerol containing 0.01% bromphenol blue, the mixtures were submitted to analysis.

migration of the protein was observed. The majority of the protein had undergone a charge alteration, being converted to more
acidic or basic forms, upon incubation with ascorbic acid and Cu(II). Little alteration of
the protein was observed in the absence of Cu(II) on both native- and SDS-polyacryl-
amide gel electrophoresis, which means that the direct reaction of the protein with ascorbic acid did not occur under the conditions used (pH 7.2, room temperature). However, when the protein was reacted with ascorbic acid in the absence of Cu(II) at 40°C, slight migration of the protein to the basic side was observed on both native- and SDS-polyacryl-
amide gel electrophoresis (data not shown).

**Effects of inhibitors on the oxidation of bovine serum albumin**

It is definite that some oxidizing species caused the structural alteration of the protein. So, the contribution of oxidizing species to the protein oxidation observed during the autoxidation of ascorbic acid was assessed through the use of a series of hydroxyl radical scavengers, enzymes and chelating agents of metal ions. As shown in Table I, catalase and EDTA completely inhibited the reactions and the Cu(II)-binding peptide, glycyl-glycyl-L-histidine, was also considerably effective. Therefore, both H₂O₂ and Cu(II) are thought to be indispensable for the oxidation of the protein. Superoxide dismutase (SOD), which promotes the generation of H₂O₂ via disproportionation of O₂⁻, showed little effect on the oxidation of the protein. Although ·OH generated via reactions 1 ~ 3 was presumed to be the principal determinant, the hydroxyl radical scavengers tested (mannitol, dimethyl sulfoxide and urea) failed to provide any protection in this system.

It cannot therefore be concluded whether or not ·OH is the major determinant in this system.

**Spectrophotometric changes of oxidized bovine serum albumin**

As the reaction proceeded, the mixtures turned light yellow in colour, and an increase in the UV absorption of the oxidized protein was observed (Fig. 4). This suggests the oxidation of amino acid residues followed by the formation of conjugated compounds. Hence, we determined the changes in the fluorescent properties of the protein to examine the loss of

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**Table I. Effects of Inhibitors on the Oxidation of Bovine Serum Albumin**

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Catalase</td>
<td>10⁴ units/25 ml</td>
<td>88</td>
</tr>
<tr>
<td>Superoxide</td>
<td>10⁴ units/25 ml</td>
<td>21</td>
</tr>
<tr>
<td>dismutase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>0.08 mm</td>
<td>93</td>
</tr>
<tr>
<td>Glycyl-glycyl-L-histidine</td>
<td>0.2 mm</td>
<td>76</td>
</tr>
<tr>
<td>Mannitol</td>
<td>10 mm</td>
<td>4</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>10 mm</td>
<td>4</td>
</tr>
<tr>
<td>Urea</td>
<td>10 mm</td>
<td>1</td>
</tr>
</tbody>
</table>

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*The reactions were carried out at room temperature, the solutions containing 0.04% bovine serum albumin, 2 mM ascorbic acid and 0.01 mM Cu(II) in phosphate buffer (25 ml, pH 7.2). Oxidation of the protein was detected by HPLC on a TSK-GEL G3000 SW column.*

*All concentrations shown are final reaction concentrations.*

*The percentage of inhibition is expressed as the inhibition rate of the decrease in the peak height of the protein (peak A in Fig. 1) on 24 hr incubation.*

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**Fig. 4. Changes in the UV Spectra of Native and Oxidized Bovine Serum Albumin.**

A, native; B, oxidized (8 hr); C, oxidized (24 hr).
Oxidation of Protein with an Ascorbic Acid–Cu(II) system

TABLE II. CHANGES IN THE FLUORESCENCE PROPERTIES OF BOVINE SERUM ALBUMIN WITH THE ASCORBIC ACID–COPPER ION SYSTEM

<table>
<thead>
<tr>
<th>System</th>
<th>Native fluorescence</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native BSA</td>
<td>694</td>
<td>100</td>
</tr>
<tr>
<td>Oxidized BSA (8 hr)</td>
<td>268</td>
<td>39</td>
</tr>
<tr>
<td>Oxidized BSA (24 hr)</td>
<td>161</td>
<td>23</td>
</tr>
</tbody>
</table>

a The native fluorescence of the protein was measured, with excitation at 280 nm and emission at 340 nm, relative to tetraphenyl butadiene, 10^{-7} M, as a standard taken as 100 units, with excitation at 350 nm and emission at 440 nm.

These data provided indirect evidence of the loss of aromatic amino acids, however, this was finally confirmed through analysis of the amino acid composition of the oxidized protein.

![CD Spectra of Native and Oxidized Bovine Serum Albumin](image)

**Fig. 5.** CD Spectra of Native and Oxidized Bovine Serum Albumin.

—○— native; —△— oxidized (8 hr); —●— oxidized (24 hr).

aromatic amino acids (tryptophan, phenylalanine and tyrosine). As shown in Table II, the results demonstrated a remarkable decrease in the native fluorescence of the protein, which indicates indirectly the loss of aromatic amino acids. We also confirmed this change by CD measurement. The absorbance at long wavelengths above 250 nm is mainly derived from aromatic amino acids, and decreases in their absorbance were ascertained (Fig. 5).

![Wavelength (nm)](image)

**Fig. 6.** Time Course of the Oxidation of Tryptophan Residues in Bovine Serum Albumin by the Ascorbic acid-Cu(II) System.

**Selective oxidation of tryptophan and histidine residues in protein**

Both the native and oxidized proteins were hydrolyzed and then subjected to amino acid analysis as described under MATERIALS AND METHODS. As shown in Table III, the loss of

![Molar ratio (%)](image)

**Table III. Comparison of the Amino Acid Compositions of Native and Oxidized Bovine Serum Albumin**

<table>
<thead>
<tr>
<th>Molar ratio (%)</th>
<th>Native</th>
<th>Oxidized</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>10.0</td>
<td>10.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Thr</td>
<td>5.0</td>
<td>6.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Ser</td>
<td>4.8</td>
<td>5.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Glu</td>
<td>15.3</td>
<td>16.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Pro</td>
<td>7.3</td>
<td>7.3</td>
<td>0</td>
</tr>
<tr>
<td>Gly</td>
<td>2.8</td>
<td>2.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Ala</td>
<td>7.9</td>
<td>8.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Cys</td>
<td>2.9</td>
<td>2.9</td>
<td>0</td>
</tr>
<tr>
<td>Val</td>
<td>6.9</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Met</td>
<td>0.6</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>2.2</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>Leu</td>
<td>10.9</td>
<td>11.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.6</td>
<td>3.4</td>
<td>-0.2</td>
</tr>
<tr>
<td>Phe</td>
<td>4.7</td>
<td>4.4</td>
<td>-0.3</td>
</tr>
<tr>
<td>His</td>
<td>2.4</td>
<td>1.1</td>
<td>-1.3</td>
</tr>
<tr>
<td>Lys</td>
<td>8.9</td>
<td>8.5</td>
<td>-0.4</td>
</tr>
<tr>
<td>Arg</td>
<td>3.8</td>
<td>3.5</td>
<td>-0.3</td>
</tr>
</tbody>
</table>

a Molar ratio (%) is expressed as the mole concentration of each amino acid per total amino acids.
histidine residues (60%) was the most prominent and the losses of lysine and arginine residues were also regarded as being significant. Of the aromatic amino acids, tyrosine and phenylalanine residues were slightly damaged, which led to the assumption that the loss of tryptophan residues might result in the changes in native fluorescence and CD of the protein. Hence, we determined the changes in tryptophan content of the protein by means of reversed-phase HPLC analysis of the alkaline hydrolyzates. The results showed that about 45% of the tryptophan residues was degraded, in proportion to the incubation time (Fig. 6). Consequently, the reaction of the protein with the ascorbic acid–Cu(II) system was accompanied by substantial losses of tryptophan and histidine residues followed by small increases in acidic amino acids.

**DISCUSSION**

The copper-catalyzed autoxidation of ascorbic acid caused the selective oxidation of tryptophan and histidine residues in bovine serum albumin. Although the mechanism underlying their oxidation has not been elucidated in detail yet, we assume that this selective reaction must be due to the ligand formation ability of histidine with Cu(II). Imidazole rings in histidine residues play a role in ligand formation with Cu(II), and they might induce the oxidative reaction due to oxygen radicals generated at a site-specific location on the protein molecule. The imidazole ring in histidine-containing peptides, in general, exhibits chelating ability with cationic metal ions under neutral conditions, and the potent reactivity of histidine might be related to such a characteristic property of the imidazole ring. Gutteridge and Wilkins suggested that Cu(II)-formed complexes with proteins could bring about site-specific damage to proteins in the presence of hydrogen peroxide, in spite of the tight binding of Cu(II) to the protein molecules. Therefore, it is reasonably possible that the complex-formation property of proteins with Cu(II) is correlated to their susceptibility to oxidation by ascorbic acid.

Thus, the reactivity of histidine can be explained by this chelating property with the copper ion, however, it is definite that the reactivity of tryptophan is due to its intrinsic reactivity with active species rather than chelation with metal ions. It is well recognized that the indole ring in tryptophan residues is considerably susceptible to oxidation at C-2 or C-3, and the formation of N-formylkynurenine and kynurenine was confirmed on radiolysis, photooxidation and reaction with lipid hydroperoxide. As to metal-catalyzed oxidation systems coupled with H₂O₂, the oxidative inactivation of superoxide dismutase was reported, and the formation of kynurenine-like compounds has been suggested. Oxidized products of tryptophan can contribute to the development of yellow and brown cataracts in the human lens, and, moreover, N-formylkynurenine and kynurenine are suspected to be promoters of urinary bladder carcinogenesis in mice. These specific reactions of tryptophan might affect the activity of the enzymes which require it for their functions.

On the other hand, the oxidation of histidine residues in bovine serum albumin by the ascorbic acid–Cu(II) system was the most prominent, however, chemical characterization of the oxidized products from histidine has been scarcely undertaken, other than in the case of the photooxidation of the imidazole ring in a histidine derivative. In some enzymes, a histidine residue is frequently located at the active center, and it functions as a chelator of metals which are essential for the enzymatic activity. Therefore, the reactions of such chelated metals with ascorbic acid or H₂O₂ must generate active oxygen radicals that cause the site-specific oxidation of imidazole rings, and this will be immediately followed by deterioration of the function of the protein.

Whether or not the oxygen-derived free radical reaction involves protein oxidation and causes site-specific modification of amino acid
residues in food and biological systems remains to be determined. Further study is needed to characterize chemically the mechanism underlying the site-specific actions of metal-catalyzed oxidation systems on tryptophan and histidine residues in relation to the non-enzymatic damage to protein in food and biological systems.

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REFERENCES